Growth, division and dna synthesis in saccharomyces cerevisiae.

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Award date:
1982

Awarding institution:
University of Bath

GROWTH, DIVISION AND DNA SYNTHESIS

IN SACCHAROMYCES CEREVISIAE

Submitted by Peter William Thompson

for the degree of PhD

of the University of Bath

1982

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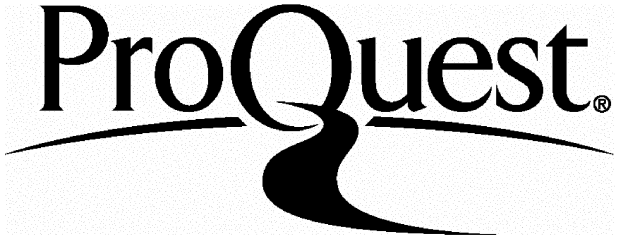


CONTENTS

ABBREVIATIONS USED

D	- Duration of daughter cycle time
P	- " " parent " "
B	- " " budded phase
τ	- Population doubling time
cdc	- Cell division cycle (gene)
G_1	- Interval from cell separation to DNA synthesis
S	- DNA synthetic period
G_2	- Interval between DNA synthesis and mitosis
M	- Mitosis
G_1*	- Interval between mitosis and cell separation
5′-dTMP	- 5′- deoxythymidine monophosphate

SUMMARY

Two different methods were employed to effect changes in the timing of cell cycle events, alteration of growth rate, and alteration of the length of S phase.

Saccharomyces cerevisiae S288C/1 was grown in a glucose-limited chemostat at population doubling times of 80 to 736 minutes. D, P and B all showed biphasic linear relationships to τ, quantitatively different from estimates for the same strain in batch culture. Median cell volume and dry weight per cell increased at the faster growth rates, but the average cell density reached a minimum at τ = 150 minutes. The contiguous array of bud scars on parent cells became increasingly irregular as τ increased from 140 minutes.

At the fastest growth rates filamentous forms constituted a small percentage of the total cell number and were presumed to arise from the failure of cells to undergo cell separation. The phenomonen was distinct from chain formation, dimorphism and pseudo-mycelial growth and showed extensive analogies with the duplication cycle of filamentous fungi.

By reducing the concentration of 5'-dTMP offered to the Saccharomyces cerevisiae tmp mutant MB1093-1D it was possible to increase the length of S phase, without altering τ. The percentage of the cell cycle S occupied could be increased from 30% at non-limiting concentrations, to over 60% at limiting concentrations.

The increase was compensated for by a reduction in G_1 and G_2. M and G_1* remained constant. At limiting concentrations P increased while D decreased.

A novel method of autoradiography using the mutant was developed.

5'-dTMP limitation also affected growth and morphology. At limiting concentrations, where τ was unaltered, up to 10% of cells had abnormal morphologies. They were of three major classes, (1) cells showing secondary bud formation, (2) cells with their bud in the centre of a "bean-shaped" cell, and (3) cells that were elongated. The length of B and volume at bud emergence increased with decreasing 5'-dTMP concentration.

CHAPTER ONE

General Introduction

GENERAL INTRODUCTION

The budding yeast Saccharomyces cerevisiae has a number of properties that have helped make it such a useful organism for research, particularly in the field of cell division. Yeast can be easily cultured, and behaves similarly in either the haploid or diploid states; mutants can be easily isolated in the haploid condition and complementation carried out in the diploid. A large number of metabolic mutants exist including a large set defective in the specific processes of cell division - the temperature sensitive cell division cycle or cdc mutants (Hartwell, 1974). These have greatly helped in the understanding of the interdependency of different cell division processes.

Yeast cells maintain a fixed shape during their cell cycle and reproduce by the budding mode. This allows the relative cell cycle stage to be determined by examining the budding morphology. When a bud is initiated a ring of chitin builds up at the isthmus (Hayashibe & Katohdo, 1973). This remains on the parent cell after the bud (now a newborn daughter) has separated from the parent and is termed a bud scar. Bud scars can be visualised by fluorescence microscopy making it possible to determine the age of a cell (Streiblova & Beran, 1963; Hayashibe, 1977). A new born daughter will have no bud scar, a parent that has budded once will have one bud scar and so on.

A disadvantage of using yeast is that the cytology is poor, particularly chromosome cytology and chromosome behaviour, which

cannot yet be analysed in detail.

Like most eukaryotic cells the yeast cell cycle is divided into four major periods, G_1, S, G_2 and M.

G_1 Phase

Control of Proliferation

The unique point of regulation of the cell cycle in Saccharomyces cerevisiae occurs in G_1 and has been termed start. Start is the earliest gene controlled event of the mitotic cell cycle (Hartwell,1974). At start, the cell monitors its environment, the presence of nutrients, the absence of conditions promoting conjugation and sporulation, the attainment of a critical size, and the completion of the previous DNA-division cycle (Nurse, 1981; Pringle & Hartwell, 1981).

Genetic control of start

Gene cdc28 is involved in the traverse of start. It is required in G_1 before the initiation of DNA replication and before the expression of any other cdc genes which function during G_1 (Hartwell, 1973a; Hereford & Hartwell, 1974). cdc28 mutants at the restrictive temperature arrest as unbudded cells and with unduplicated spindle plaques (Byers & Goetsch, 1975). New start mutants have now been isolated (Reed, 1980).

α - Factor arrest at start

When actively growing cultures of opposite mating type are

mixed their cell cycles become synchronised in G_1 prior to cell fusion by the mating pheromones that are produced and secreted by cells of each mating type (Hartwell, 1973b). α factor is produced and secreted by cells of mating type α and arrests a cells, and vice versa (Betz & Duntze, 1979). The cells arrest in G_1 prior to bud emergence, initiation of DNA synthesis and spindle pole body duplication (Throm & Duntze, 1970; Hartwell et al, 1974; Byers & Goetsch, 1975). During conjugation, cell agglutination is followed by cell and nuclear fusion and a mitotic cell cycle.

Reid & Hartwell (1977) have shown that cells arrested at the cdc28 step can undergo conjugation whereas cells arrested at later cdc gene steps cannot. This is evidence for mating factor arrested cells being at the same stage of the cell cycle as the expression of the cdc28 gene (Hereford & Hartwell, 1974).

Regulation of size at start

During exponential growth, yeast cells have a narrow and characteristic range of cell sizes. Control mechanisms must exist to co-ordinate growth and division. A model was proposed by Johnston, Pringle and Hartwell (1977) and developed by Hartwell and Unger (1977).

The model has two basic proposals, (1) that growth rather than progress through the DNA-division cycle is rate-limiting for cell division, i.e. cells can complete the DNA division cycle faster than they can double in mass, and (2) that growth to a

critical size is a prerequisite for the completion of at least one step in the DNA-division cycle, the start event. It is not known exactly what is meant by size, it is presumably a molecular species, whose amount or concentration is coupled to cell volume, or mass, or protein content, or RNA content all of which increase in parallel in steady state cultures (Pringle & Hartwell, 1981; Johnston & Singer, 1981; Wheals & Silverman, 1982; Fantes et al, 1975).

Upon nitrogen starvation, cells that have passed start can divide with less than a 10 percent increase in cellular protein and produce abnormally small daughters. When reinoculated into fresh medium, the time before the small cells bud is inversely related to their initial volume (Johnston, Pringle & Hartwell, 1977). The experiment shows that completion of the cell division cycle after start does not require significant growth and that traverse of start does require growth to a critical size.

Bud emergence occurs shortly after start and can be used as a marker for the initiation of a DNA-division cycle.

The fact that cdc mutants blocked at various stages of the cell cycle are able to continue growth and achieve volumes, masses and protein contents 2-3 times those of exponentially growing cells, is evidence that there is no control limiting growth in the absence of division. Strong support for the basic tenets of the model have come from, Jagadish & Carter (1977); Slater, Sharrow & Gart (1977); Tyson, Lord & Wheals (1979); and Lord & Wheals (1981).

The relationship between growth and division in Saccharomyces cerevisiae is emphasised by the fact that the division of budding yeast is asymmetrical - at all growth rates the new (daughter) cells are born smaller than the old (parent) cell. As the growth rate decreases this difference is greater (Beran, 1968; Hayashibe & Sando, 1970; Vrana, 1976; Hartwell & Unger, 1977; Carter & Jagadish, 1978a; Lord & Wheals, 1980). The daughters born at a smaller size require a longer period of growth before reaching the critical size necessary for initiation of the DNA-division cycle. Mother cell volume increases during the cell cycle and therefore they must be born greater than the critical size and need not undergo an extensive period of growth before initiating start. Consequently differences in cycle times between mothers and daughters are observed. Equations have been derived to determine cell cycle age distribution (Hartwell & Unger, 1977), mean cell age and volume (Tyson, Lord & Wheals, 1979) and the genealogical age distribution (Lord & Wheals, 1980) in exponentially growing populations.

Shilo and his co-workers have measured the kinetics with which cells complete start and found them to be approximately first order, suggesting that traverse of start is probabilistic (Shilo, Shilo & Simchen, 1976; Shilo, Simchen and Pardee, 1978; Shilo, Ridde & Pardee, 1979) similar to mammalian cells (Smith & Martin, 1973; Brooks, Bennett & Smith, 1980). Controversy has arisen over this issue (Nurse & Fantes, 1977; Wheals, 1977; Shilo, Shilo & Simchen, 1977). Nurse (1980) has proposed that both deterministic and probabilistic events occur at start, and Wheals (1982) has

expanded this idea and has experimental evidence to support models for control of start in which yeast cells include a strongly deterministic element relating to cell size together with a sloppy size-monitoring mechanism that permits a degree of randomness in the behaviour of cells of similar sizes.

Nutrient regulation of start

Cells deprived of carbon and energy, a nitrogen source, sulphate, phosphorus, potassium and biotin arrest before bud emergence and the completion of the cdc28 function (Hartwell, 1974). Cells leave the mitotic cycle and enter stationary phase prior to traverse of start.

Unger & Hartwell (1976) suggested that the signal for monitoring all nutrients was generated at the level of protein synthesis. Johnston & Singer (1981) proposed an integrated sequence of responses of at least three steps.

The first is some aspect of protein synthesis similar to the proposal of Unger & Hartwell (1976). A second step is the production of ribosomal precursor RNA (rpreRNA). RNA synthesis inhibitors cause cells to accumulate at start (Johnston & Singer, 1978; Bedard, Singer & Johnston, 1980; Singer, Johnston & Bedard, 1978; Singer & Johnston, 1979). Although there is little effect on mRNA and SRNA production, there is a significant decrease in the rate of rpreRNA production and its processing to yield mature RNA. By the use of rna mutants Johnston & Singer (1980) showed

that it is rpreRNA production that is being controlled. The third step is start itself, the stage sensitive to mating pheremone. G_1 arrest by mating pheremone should not affect protein or RNA synthesis and this is the case (Throm & Duntze, 1970).

Chromosome behaviour at start

Folded chromosomes can be isolated from yeast which change their sedimentation profiles as they progress through the DNA-division cycle (Piñon & Salts, 1977) and characteristic G_1 and G_2 forms can be identified. Stationary phase cells starved of nitrogen have a characteristic G_0 form (Piñon, 1978) and α factor arrested cells show another form, even though both treatments result in arrest at start (Piñon & Pratt, 1979). α factor arrested cells can enter stationary phase and vice versa, without entering a further mitotic cycle (Piñon & Pratt, 1979). They proposed that cells leave the cycle at start and, depending upon the conditions causing arrest, enter a specific reversible pathway. Interchange between pathways within a start "area" is possible if the conditions change. In an a/α diploid the stationary phase pathway can be extended into sporulation.

Length of G_1

Altering the growth rate by nutritional limitation or other means changes the intervals of the cell cycle (Mitchison, 1971). Results comply with the idea that most of the increase in cycle time that occurs when the growth rate is slowed occurs in the interval from division to start, i.e. when cells are trying to reach the

critical size.

The interval from bud emergence to division, completion of DNA synthesis to division, initiation of DNA synthesis to division, execution of cdc28 and cdc7 functions to division and the interval from the execution point for mating factor response to division, have all been reported to be constant with generation times from 2-6 hours (von Meyenburg, 1968; Slater, Sharrow & Gart, 1977; Jagadish & Carter, 1978; Barford & Hall, 1976; Carter & Jagadish, 1978b; Jagadish & Carter, 1977).

Criticism can be levelled at some of the analyses. For example, (1) Saccharomyces cerevisiae suffers from a weak genomic signal when flow microfluorimetry is used and clear definitions of cell cycle phases are difficult (Slater, Sharrow & Gart, 1977), (2) The wrong equation has been used (von Meyenburg, 1968), and (3) Interpretation of autoradiograph results also needs the correct analysis (Rivin & Fangman, 1980a).

Where the correct equation for the length of the budded phase (B) is used, there is an increase, however slight, in B with an increasing population doubling time and this increase is linear (Wheals, 1982b; Tyson, Lord & Wheals, 1979; Lord & Wheals, 1981). Most variation was still in the interval from division to bud emergence.

The increase in B with increasing growth rate indicates that

the rate of accumulation of some stage-specific components not present in excess can be rate limiting under conditions of slow growth (Pringle & Hartwell, 1981). The volume at which bud initiation occurs varies with growth rate and cell age. As the growth rate increases, cell volume at bud initiation decreases showing a characteristic J shaped curve (Johnston, Ehrhardt, Lorincz & Carter, 1979; Lorincz & Carter, 1979; Tyson, Lord & Wheals, 1979; Lord & Wheals, 1980). The mother cell volume increases by about ten percent at each generation (Hartwell & Unger, 1977; Lorincz & Carter, 1979; Johnston et al, 1979). Shift up and shift down experiments have shown that cells regulate their volume in G_1 close to start (Johnston et al, 1979; Lorincz & Carter, 1979).

A report by Rivin and Fangman (1980a) found that as the growth rate increases the proportion in G_1, S and G_2 remained constant at 0.25, 0.50 and 0.25 respectively. Nitrogen limitation may be producing a different response, or perhaps basic amino acid inhibition of DNA synthesis occurred.

G_1 in Other Organisms

G_1 is also the most flexible cell cycle stage in other cell types. On average mammalian cells have doubling times of 18-20 hours with a G_1 of 8-10 hours. Cell types with a shorter doubling time have a shorter G_1 and those growing more slowly have a longer G_1 (Mitchison, 1971). This is the case for cells of the same tissue grown at different rates (Aoki & Moore, 1970), and when

cycle time is limited by nutrient supply (Tobey, Anderson & Petersen, 1967; Gautschi, Schindler & Hurni, 1971).

The control of proliferation in mammalian cells is very much an area of conflicting ideas - for a review see Yanishevsky and Stein (1981). There is contradictory data on a size-related requirement at or before the initiation of DNA synthesis. Cell fusion experiments suggest that in cycling cells entry into S phase is positively controlled, while in senescent cells there is also a negative control mechanism (Yanishevsky & Stein, 1981). Smith and Martin (1973) proposed a totally different model, the Transition Probability Model of cell proliferation which stated that the cell cycle has a constant B phase from the initiation of DNA synthesis to division, and an A-state, in G_1, from which cells have a constant probability per unit time of leaving and re-entering B. The model has consequently been modified (Brooks, Bennett & Smith, 1980). Smith, Laurence and Rudland (1981) have since suggested that attempts to deduce mechanisms consistent with kinetic data are pointless since many models can be adjusted to accept new data. Their conclusion is false, and important data can be gleaned if other parameters are measured together with the kinetic data, or if perturbed cells are used. Further experimental work is required to clarify the confused picture of regulation in mammalian cells.

In Schizosaccharomyces pombe commitment to the DNA-division cycle is located in G_1 and is controlled by the gene products of

cdc2 and cdc10 (Nurse & Bissett, 1981). Completion of start occurs at the beginning of the cell cycle in rapidly growing cells, but is delayed to later in the cell cycle at slower growth rates (Nurse, Thuriaux & Nasmyth, 1976; Nasmyth, 1979).

Preparation for S Phase

G_1 cells are involved in preparation for S phase and a number of different S related activities have been identified. Walters, Tobey & Ratliff (1973) showed that enlargement of the precursor pool of deoxyribonucleotide triphosphates began in late G_1 in CHO cells. Nexø (1975) has similar results for Tetrahymena pyriformis. Gurley, Walters and Tobey (1974) found histone H1 phosphorylation began in the latter half of G_1 in CHO cells. Some of the proteins made in G_1 are presumably enzymatic components of the replication complex since inhibition of G_1 protein synthesis prevents the initiation of DNA synthesis. In Saccharomyces cerevisiae addition of cycloheximide up to a critical point 10 minutes before start of S prevents entry into S (Williamson, 1974).

The Nature of G_1

The view that during G_1 a sequence of cell cycle specific events must occur to prepare for DNA synthesis has been questioned by Cooper (1979) and Liskay (1978). A CHO line V79-8 lacks a detectable G_1 phase in its cell cycle (G_1^-). Using somatic cell hybrids, results show that the G_1^- phenotype is dominant in intra-specific crosses but recessive in interspecific crosses. G_1^- V79-8 complements at least two different G_1 deficient cell cycle

mutants, suggesting that it retains at least some G_1 specific functions (Liskay, 1978). Cooper (1979) and Liskay (1978) propose that G_1 is present not because events specific to G_1 are taking place but because events specific to growth have not yet occurred in sufficient quantities. Singer and Johnston (1981) have experimentally tested this idea in Saccharomyces cerevisiae and their results partially agree with this proposal. Their work complements that discussed in Chapter 4 and will be analysed in further detail there.

In summary G_1 is characterised by a large variation in length that accounts for most of the increase in cell cycle length, is the point of regulation of the cell cycle, and undergoes activities to prepare the cell for DNA synthesis.

S Phase

Saccharomyces cerevisiae, like other eukaryotes replicates its genome periodically during a distinct portion of the cell cycle, the S phase. To maintain ploidy each chromosome must be replicated once and the daughter chromosomes correctly apportioned to each cell.

RNA transcription continues during S phase of many types of cells (Mitchison, 1971) and has been observed on both daughter strands during replication in Drosophila (McKnight & Miller, 1977). The disruption of the chromosomes by the passage of the replication fork may alter the control of the replicating genes. This may be a factor in the regulation of histone genes which are expressed in S

in many types of cells (Edenberg & Huberman, 1975) including Saccharomyces cerevisiae where the synthesis of histone mRNA is tightly and co-ordinately regulated with the replication processes (Hereford et al, 1981). Gene dosage is also transiently altered during S and this may be important in the timing of cell cycle events.

DNA Structure

The amount of DNA per haploid genome in Saccharomyces cerevisiae is 9×10^9d or 1.4×10^4kb (Lauer, Roberts & Klotz, 1977; Bicknell & Douglas, 1970) and the number of linkage groups is 17 (Mortimer & Hawthorne, 1973). Estimates of the size of chromosomal DNA range from 5×10^7 - 1.4×10^9 daltons (Petes & Fangman, 1972) and 4×10^8 - 6×10^8 daltons (Blamire et al, 1972), the average molecular weight was calculated to be 6×10^8 daltons (Petes & Fangman, 1972).

Yeast chromosomes contain basic proteins with the same electrophoretic mobilities as the histones H2a, H2b, H3 and H4 of higher eukaryotes. It is uncertain whether yeast has a histone comparable to H1, a yeast protein with similar electrophoretic properties has been found, however the ratio of lysine to arginine was considerably less than that for calf thymus H1 (Thomas & Furber, 1976; Suchiliene & Gineitis, 1978; Sommer, 1978). Micrococcal and staphylococcal nuclease digestion has shown that yeast nuclear DNA is packaged as nucleosomes. The amount of DNA between adjacent yeast nucleosomes was found to be about 20 base pairs, somewhat less

than that observed for most higher eukaryotes (Kornberg, 1977). DNase I digestion of yeast chromatin indicated that the arrangement of DNA within the nucleosome was similar to that of higher eukaryotes (Thomas & Furber, 1976; Lohr et al, 1977).

DNA Replication

Yeast chromosomes initiate DNA replication from multiple replication origins between 10-40 μm apart (Newlon et al, 1974; Petes & Williamson, 1975b). This is similar to values in mammalian cells (Edenberg & Huberman, 1975). With an average inter-origin distance of 30 um, and the average yeast chromosome being 230 μm (Petes, Byers & Fangman, 1973) there will be 8 origins per chromosome and 136 in the haploid genome. This may be an under-estimate as replicating chromosomes observed by electron microscopy or auto-radiography may contain origins that have not yet been activated (Petes, 1980). DNA replication is bidirectional from each initiation site and the rate of fork movement at 24°C was 0.7×10^6 daltons/min. per replication fork (Petes & Williamson, 1975a). In a different diploid strain at 25°C it was 0.82×10^6 daltons/min. (Johnston & Williamson, 1978) while in a haploid strain at 30°C a rate of 2.1-0.56 μm/min. ($3.78 - 1.0 \times 10^6$ daltons/min.) was reported (Rivin & Fangman, 1980b).

In higher eukaryotes and prokaryotes DNA is synthesized initially as small "Okazaki" fragments (Edenberg & Huberman, 1975). In experiments in which yeast cells were labelled for 2 mins., short single-stranded DNA molecules were observed (Johnston & Williamson,

1978). Johnston and Nasmyth (1978) isolated DNA from a temperature sensitive yeast mutant defective in the DNA ligase enzyme and found that single-stranded DNA fragments accumulated at the restrictive temperature but not the permissive. It should be noted that small single-stranded DNA fragments can be generated by misincorporation and excision of uracil, so the exact nature of these fragments remains uncertain.

Control of DNA Synthesis

The control of DNA synthesis and its co-ordination in the cell cycle has been studies by Hartwell and his co-workers (Hartwell, 1971a; Hartwell, 1976). He isolated temperature sensitive mutants defective in DNA synthesis; cdc8 and cdc21 were classified as elongation mutants and cdc4, 7 and 28 as DNA initiation mutants. The initiation mutants, although required for initiation of S phase may not be directly involved in the process (Hereford & Hartwell, 1974). cdc21 has been shown to be defective in the enzyme thymidylate synthetase (Game, 1976; Bisson & Thorner, 1977). The mutant cdc8 is necessary for error-prone repair although it does not code for a DNA polymerase (Prakash, Hinkle & Prakash, 1979) and cdc9, originally thought to be a nuclear division mutant, is defective in DNA ligase (Johnston & Nasmyth, 1978). Considering the number of proteins known to be involved in DNA replication it is surprising that so few DNA synthesis mutants are known, and studies are now underway to isolate more possible mutants (Thomas & Johnston, 1981).

Results obtained with pulse treatment of synchronized yeast

cultures with nitrosoguanidine (Burke & Fangman, 1975; Sim & Haber, 1975) where different loci were mutagenised at different times during S phase, and the observation that some yeast chromosomal segments when inserted into a recombinant DNA plasmid and transferred back into a yeast cell were capable of autonomous replication while others were not (Hinnen, Hicks & Fink, 1978; Struhl et al, 1979), indicate that the DNA replication origins are defined sequences on the chromosome and that individual replication origins replicate in the same order in S phase in different cycles; as is found in mammalian cells (Adegoke & Taylor, 1977).

Extrachromosomal DNA

The other gene systems in Saccharomyces cerevisiae have also been studied. Many strains contain small extrachromosomal DNA molecules 2 μm in length, the 2 μm plasmids. There are between 50-100 copies per cell and each copy replicates once in the cell cycle, during the S phase (Zakian, Brewer & Fangman, 1979). They require the same gene functions for replication as chromosomal DNA (Petes & Williamson, 1975; Livingston & Kupfer, 1978). Mitochondrial DNA synthesis is continuous throughout the cell cycle (Sena et al, 1975) and requires the gene functions for DNA elongation but not DNA initiation (Newlon & Fangman, 1975). A new class of extrachromosomal DNA, 3 μm DNA, has been discovered by Larianov, Griskin and Smirnov (1980). It is a chromosomal repetitive unit of rDNA. Nothing is yet known of its replication.

Length of S Phase

The length of S phase in Saccharomyces cerevisiae has been measured using a variety of techniques and under a number of growth conditions.

Williamson (1965) using a diploid strain reported that incorporation of labelled adenine into DNA was almost exclusively during the first quarter of the cell cycle. The initiation of DNA synthesis occurred at the same time as bud emergence, and S phase lasted for 27% of the cell cycle, 32 minutes of a 120 minute doubling time.

Hartwell (1970) used the rate of incorporation of labelled uracil to estimate S phase in a diploid strain synchronized by renograffin-sucrose density gradients. S phase occupied 30% of the cell cycle, lasting 40 minutes of a 135 minute doubling time. Bud emergence occurred midway through S.

Barford & Hall (1976) measured S in a diploid strain by counting the fraction of cells labelled in a pulse of ^{3}H adenine. They varied the growth rate by changing the carbon source and found S to be 45 minutes long in cultures with doubling times of 92, 181 and 435 minutes. Bud emergence occurred shortly before the initiation of DNA synthesis.

Flow microfluorimetry has also been utilised to estimate the length of S. Slater, Sharrow & Gart (1977) found that S was constant

at 20 minutes in a diploid strain with cycle times between 85-258 minutes. Bud emergence and DNA initiation were indistinguishable. Variation in the growth rate was achieved by changing the carbon source and other nutritional factors. A similar analysis by Johnston et al (1980) on the same strain, where growth rate was varied by changing the nitrogen source, again showed bud emergence and DNA synthesis to be indistinguishable, but there was a four fold increase in S from 18-72 minutes with a cycle time increase of 144-456 minutes.

Rivin and Fangman (1980a) using three different autoradiography techniques in nitrogen limited cultures found that, regardless of the nitrogen source or method of estimation, the S phase occupied approximately 50% of the cell cycle. In this haploid strain bud emergence occurred halfway through S.

Despite the number of methods employed, or perhaps because of them, no clear cut picture of the length and variability of S phase in Saccharomyces cerevisiae exists.

Experiments with the drug hydroxyurea have further confused the picture. Slater (1973) and Hartwell (1976) found that all the budded cells in an exponential culture completed a round of DNA replication in the presence of hydroxyurea. The drug normally produces a rapid and specific inhibition of DNA synthesis, therefore DNA synthesis must have been completed by the time of bud emergence.

From the data a general picture of a shorter S phase in diploids compared to haploids emerges. Williamson (1981) suggested that the difference could be due to all replicons firing at the start of S in diploids, while in haploids replicons fire throughout S. In pre-meiotic DNA synthesis the S phase lasted an average of 65 min. Replicon size and rate of fork movement were the same as in mitotic cells, at 28 μm and 0.7 μm/min. respectively, therefore the average replicon must be completed in about 20 min. Origin activation in the meiotic cells is considerably staggered, (Williamson, personal communication). There is recent evidence which contradicts this hypothesis. Fangman (personal communication) has observed that a diploid has a longer S than its isogenic haploid. He also found that S phase length can differ significantly between mothers and daughters.

Length of S Phase in Other Organisms

Among mammalian cells the S phase remains constant in duration between cells of different species or of different tissues, 6-8 hours of an 18-20 hour cycle time. A few tissues have a longer S phase of 12-13 hours (Mitchison, 1971). Human lymphoid lines (Aoki & Moore, 1970), CHO cells (Tobey, Anderson & Peterson, 1967) with different cycle times and murine cells grown at different rates due to nutritional limitation (Gautschi, Schlinder & Hurni, 1971), showed little variation in S phase length. Killander and Zetterberg (1965) found that in 21 mouse L cell lines the length of $S + G_2$ was inversely related to G_1, ranging from 4-18 hours.

Cells in different developmental stages show large differences in the length of S. Cultured cells of D. melanogaster require 10 hours to complete S (Dolfini, Courgeon & Tiepolo, 1970) whereas cleavage nuclei divide every 9.6 minutes with only 3.4 minutes of interphase (Blumenthal, Kriegstein & Hogness, 1973). Callan observed similar results with the newt Triturus (Callan,1972; 1973). Somatic cells at 25°C require 2 days to traverse S, while cells of the neurala take only 2 hours. Spermatocytes at 18°C need a premeiotic S of 9-10 days. Snow (1977) found mouse embryos with a total cycle time of two hours, considerably less than the minimum S phase previously reported of 6 hours.

Cause of Variation in S

Clearly large variation in the time required for DNA replication exists and there are processes which could be rate determining for S, replicon initiation and spacing, and fork rate.

Callan (1972; 1973) measured fork rate and interorigin distance of replicating DNA from somatic, neurala and spermatocyte cells. In the spermatocytes replicons were too far apart for tandem origins to be measured, in the somatic cells they were 100 microns apart and in the neurala 40 microns. Fork rates were similar on each cell type. The shorter the S phase the closer the tandem origins, S phase can be altered by activation of different sets of origins. Blumenthal et al (1973) achieved similar results between cleavage nuclei of Drosophila and cultured cells. Fork rates were comparable but cultured cells had a four-fold greater interorigin distance.

This does not account for such a vast difference in S, and the authors proposed that rate-limiting adjacent origins are so far apart that strand breakage prevents them being measured.

Activation of new replicons through S has been advanced to explain the continuous requirement for protein synthesis during DNA replication in many eukaryotes. Muldoon et al (1971) divided the S period in Physarum polycephalum into ten stages each with a requirement for protein synthesis. Recent work by Evans et al (1976) has cast doubt on this interpretation. Continuous protein synthesis is not necessary for completion of S in Saccharomyces cerevisiae (Hereford & Hartwell, 1973; Williamson, 1973) and Petes and Williamson (1975a) proposed that all replication origins in yeast are activated early in S. Care should be taken in analysing the results of experiments where protein synthesis is inhibited as the effect depends upon the inhibitor, the dose and the type of cell (Stimac, Housman & Huberman, 1977).

Eukaryotic cells exhibit replication fork rates ranging from 0.2 - 4.0 microns/min. (Edenberg & Huberman, 1975). Rates of DNA synthesis can change during S phase (Klevecz, Keniston & Deaven, 1975; Collins, 1978). This could be due to the activation of new replicons, but measurements have shown that fork rates in mammalian cells vary 2 or 3 fold during S. (Housman & Huberman, 1975; Painter & Schaeffer, 1971). The patterns of replication during S depends on the cell type, and unfortunately also on the method of analysis (Collins, 1978).

In Saccharomyces cerevisiae Rivin and Fangman (1980b) found that fork rates were inversely proportional to the length of S phase and that fork rate variation could account entirely for the S phase variation. Fork rate was constant throughout S. There was no changes in origin to origin distances, adjacent origins were activated within a few minutes of each other, and new activations occurred throughout S.

From the different experiments it is clear that S phase is a highly ordered and regulated programme of events, yet there is pliability within the system. Origin spacing can be changed during development and fork rate changed when the growth rate is nutritionally limited. The new approach in Saccharomyces cerevisiae of selective lengthening of S phase, without altering the doubling time, by inhibitors (Singer & Johnston, 1981), or limiting thymidylate to thymine requiring mutants (this work), should lead to a further understanding of the control of S and its co-ordination with the rest of the cell cycle.

G_2 and M Phases

G_2 is the stage in the cell cycle when the nuclear DNA content has doubled and the chromosomes are uncondensed. M is the stage where the chromosomes condense and are partitioned equally between the mother and bud. The cessation of incorporation of radioactive precursors into chromosomal DNA marks the S/G_2 boundary. The G_2/M boundary is difficult to determine and it depends upon when the chromosomes are judged to be in prophase. Chromosome

condensation cannot be observed in yeast and nuclear division morphology is used to mark the end of G_2. In most cell cycle analysis M is not measured separately but included with G_2.

In Saccharomyces cerevisiae Slater et al (1977) found the G_2, M and G_1* interval increased two fold where the increase in doubling time was three fold, growth rate was changed by altering the carbon source and other nutrients. When the growth rate was altered by nitrogen limitation the results were the same (Johnston et al, 1980). Barford and Hall (1976) observed a three fold increase in G_2 with a five fold increase in doubling time, whereas M was constant.

The length of G_2 varies according to the species and tissue origin. Among amphibia, very early embryos have no G_2 at all, but after gastrulation it can account for over 50% of the cell cycle (Graham & Morgan, 1966). In different mammalian tissues G_2 ranges between 0.5 - 5.0 hours (Mitchison, 1971). Variation in mammalian cell doubling time accomplished by using different serum lots (Tobey et al, 1967) or limiting aminoacids. (Gautschi et al, 1971) show a constant G_2. It is also constant in individual cell lines of the same type with different doubling times (Aoki & Moore, 1970). When greater alterations in growth rate are achieved by glucose limitation G_2 is significantly increased.

Rao and Johnson (1974) in their studies on Hela heterokaryons found that G_2 nuclei could be characterised by susceptibility to

condensation upon fusion with a mitotic cell, and insensitivity to re-initiation of DNA synthesis when fused with late G_1 or S phase cells. G_2 nuclei could shorten the G_2 of another nucleus. The results imply that proteins necessary for chromosome condensation are made during G_2 and that factors must be present in the nucleus that counteracts inducers of DNA synthesis.

The G_2 phase prepares the cell for nuclear division and is variable in length when growth is limited, although on a much smaller scale than G_1.

There is strong evidence that RNA synthesis is very much reduced in mitotic cells, presumably because DNA cannot be transcribed when the chromosomes are in a highly condensed state. In some cells protein synthesis is also reduced, in others it continues at an unaltered rate.

All previous data are for populations. It is now clear (Lord & Wheals, 1981) that there is also considerable variability between individual cells, knowledge of which must also be integrated into our understanding of control of the cell division cycle.

Two methods of investigating the control and co-ordination of the cell cycle have been neglected. Firstly, examination at

extremely fast and extremely slow growth rates, and secondly, the effects of altering the length of a cell cycle phase without altering the population doubling time. The aim of the project was to study in detail both of these areas.

CHAPTER TWO

Materials and Methods

MATERIALS AND METHODS

CHEMOSTAT

Organism A wild type haploid strain of Saccharomyces cerevisiae obtained from Dr. C.F. Roberts, Genetics Department, University of Leicester, was used for the chemostat analysis. A derivative of S288C, it is designated S288C/1 (Lord & Wheals, 1980).

Medium YEPD medium was used; it contained 10g yeast extract, 20g bacteriological peptone and 20g glucose per litre. It was filtered twice through Whatman GF/D and GF/F filters to remove any particles, and then sterilised by autoclaving.

Growth conditions Continuous cultivation was carried out in a cc 1500 fermenter (L.H. Engineering, Stoke Poges, Bucks.) using a working volume of 2 litres. The temperature was maintained at $30^{\circ}C$, the air flow at 2 l min^{-1}, the stirrer speed at 600 rev min^{-1}, and the pH at 5.5 regulated by the addition of 1M-HCL or 1M-KOH. Foam formation was reduced by the addition of a 5% (v/v) solution of silicone DC antifoam emulsion M10 (Hopkin & Williams, Chadwell Heath, Essex). After a change in the dilution rate (equal to μ), the culture equilibriated after 20 l of the medium had passed through the vessel and new measurements were then taken. Samples were fixed with a saline formaldehyde solution containing 0.9% NaCl and 4% formaldehyde and briefly sonicated to disperse clumped cells.

Bud scar analyses Bud scar analysis was carried out on samples of cells which were in the mid exponential phase of growth. Where necessary, cell suspensions were concentrated by collection on a microfilter. The cells were stained with a 2 mg/ml solution of calcoflour (a gift from J. Peberdy). Cells were viewed at X 1,250 by using incident UV light with appropriate filters and a Leitz Orthoplan phase-contrast microscope. The number of cells in each of the following categories were determined: (1) unbudded daughters, (2) budded daughters, (3) unbudded parents with n scars, and (4) budded parents with n scars. The number of budded cells and total scars was determined. At least 1,000 cells were scored at each growth rate.

Cell number and cell volume measurements Cell counts and volume were determined by using a model 111 LTS Electrozone/Celloscope (Particle Data Inc., Elmhurst, Ill.) fitted with a 60-um orifice tube. Cell volume distributions were obtained by using a Nuclear Data Model 1100 Analyser System (Nuclear Data Inc., Palatine, Ill.) coupled to a Hewlett-Packard X-Y plotter (Hewlett-Packard Inc., Pasadena, Calif.). Median cell volumes were obtained from the peaks of the normal distribution of volumes (on a log scale). The equipment was calibrated by using standard 5.7 μm diameter latex spheres (Dow Chemical Co.).

Dry weight Determination A 5 ml culture sample was filtered through a pre-dried, pre-weighed, 0.45 μm pore-size cellulose acetate Nuflow Filter (Oxoid), washed several times in distilled H_2O

and dried to a constant weight in a vacuum drying oven at 120°C. Five samples were taken at each growth rate.

Estimates of D, P and B These were calculated by the maximum likelihood method from the bud scar analysis data and the dilution rate (equal to $\ln 2/\tau$) as described previously (Lord & Wheals, 1980).

Genealogical age distribution The predicted frequency of cells at different genealogical ages can be calculated by substituting estimates of P into,

$$F_D = e^{-\alpha P}$$

for the fraction of daughter cells and

$$F_{P(\underline{n})} = (e^{-\alpha P})\, \underline{n}^{-1}\, (1 - \alpha^{-\alpha P})^{2}$$

for the fraction of parent cells of genealogical age $\underline{n}$ where $\underline{n}$ is equal to the number of bud scars (Lord & Wheals, 1980).

Enzyme treatment Filamentous cells were treated with Zymolyase 5000 (Kirin Brewery, Takasaki, Gumma Pref., Japan), at a concentration of 0.04 mg. Zymolyase per mg. dry weight of yeast for 2 to 3 hours at 23°C (10% of the concentration needed to spheroplast the cells in the same time).

5'-dTMP EXPERIMENTS

Organism The strain used, MB1093-1D, was supplied by Martin Brendel (Fachbereich Biologie der Johann Wolfgang von Goethe-Universitat, Institut Fur Mikrobiologie, 6000 Frankfurt/Main,

Federal Republic of Germany). It was a haploid, petite, a mating type strain of Saccharomyces cerevisiae which was a low requiring thermosensitive 5'-dTMP auxotroph, capable of uptake of 5'-dTMP (Brendel, 1976).

Medium Medium N contained 6.7 g Difco yeast nitrogen base without amino acids, 2.0 g Difco casaminoacids without vitamins, 20g of glucose, and 0.05g of both adenine and uracil, added to 1 litre of distilled water. Where appropriate Agar (oxoid No. 3) was added at a concentration of 20g l^{-1}. The medium was filtered to give particle free solutions and sterilised by autoclaving, except for the yeast nitrogen base which was filter-sterilised. YEPD medium used as described above had adenine and uracil added at concentrations of 0.05g l^{-1}.

5'-dTMP was obtained as the disodium salt (Sigma). Medium N was supplemented with 5'-dTMP from a concentrated filter-sterilised stock solution.

Growth conditions Cells were grown, either at the permissive temperature of 23°C or the restrictive temperature of 36°C, with shaking in 50-100 mls of medium in 250 ml Erlenmeyer flasks. Samples were fixed with a solution of saline formaldehyde and briefly sonicated to disperse clumped cells. Growth was determined by total cell count.

Cell number and volume measurements Cell counts were determined as

described above. Cell volume at bud emergence was analysed by a Watson image-shearing eye piece (Vickers, England). At least 50 cells were counted for each genealogical age at each concentration.

Alpha factor The alpha factor was supplied by the Peptide Institute Inc., Osaka, Japan. It was used at a concentration sufficient to keep 10^8 cells at the block point for one generation time.

Bud scar analyses, Estimates of D, P and B These were carried out as previously described (see above).

Nuclear staining The fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI) (a gift from Don Williamson) was used to visualise nuclear morphology. The dye was added to 10^7 cells at a concentration of 0.5 µg/ml., left overnight at 4°C, and the cells then examined under UV light (Williamson & Fennel, 1975). At least 1000 cells were analysed at each 5'-dTMP concentration.

Isotope Whole cell autoradiography employed (methyl- ^3H) Thymidine 5'-monophosphate, ammonium salt 45 Ci/m M (Amersham International Ltd.).

Preparation for whole cell autoradiograms Asynchronous cultures were grown to mid-exponential phase ($4-7x10^6$ cells ml^{-1}) at 36°C, and radiolabelled 5'-dTMP added to the required radioactive concentration. After 15 minutes samples were taken for autoradiography,

and fixed in saline formaldehyde. Cells were then filtered, washed and left to stand at 4°C for 30 minutes in 10% trichloroacetic acid (TCA). The cells were collected on membrane filters and washed five times with ice-cold phosphate buffer. The cells were then resuspended to a concentration of 10^6 cells/ml. 0.1 of the cell suspension was added to microscope slides that had been cleaned with acetone and then coated with a thin film of egg albumen. The sample was spread across the slide and air dried.

The dry slides were dipped in Ilford K_2 emulsion at 43°C that had been diluted 1:1 with 2% glycerol. The emulsion was allowed to dry flat for 45 minutes, and then stored in sealed slide boxes at 4°C. After exposure of 3-6 weeks the slides were developed for 6 minutes in a 1:5 dilution of Ilford phenisol, washed twice in distilled water, fixed for 8 minutes in a 30% sodium thiosulphate solution, and finally washed for 15 minutes in running water. Slides were then examined microscopically for the presence or absence of grains.

RNase was added at a concentration of 10 mg/ml for 45 minutes at 37°C, DNase was added at a concentration of 1 mg/ml.

CHAPTER THREE

Chemostat Work

INTRODUCTION

Analysis of the cell cycle via manipulation of the growth rate is a useful method for understanding temporal controls. One method for altering the growth rate is by changing the flow rate of a limiting nutrient in a chemostat. The major advantages of a chemostat are, that the growth medium is the same over the growth range used, and that the cell cycle can be examined at extremely fast and extremely slow growth rates, where normal control methods might be stretched to the limits.

Despite this the chemostat is a much underused apparatus in cell cycle analyses of Saccharomyces cerevisiae. Variation in cell cycle times by glucose-limited chemostat has been accomplished, although no detailed analysis has been made (von Meyenberg, 1968; Jagadish & Carter, 1977; 1978; Carter & Jagadish, 1978). The implicit assumption has been that the results are equivalent to those for batch culture.

Very interesting observations have been noted with Candida utilis. During hypertrophic growth in a two-stage fermenter secondary bud formation was seen. This would occur if the doubling time was so fast that there was insufficient time for completion of the budded phase, in this case a second bud would appear on the parent before the first bud has separated (Vrana, 1973). Interestingly they also claim that some cells during hypertrophic growth do not contain DNA, although they have reached the size of their mother cells or had another daughter cell (Vrana, Lieblova & Beran, 1973).

The aims of the work reported here were to use a chemostat to, (1) analyse the cell cycle of Saccharomyces cerevisiae over a wide range of growth rates, (2) to examine hypertrophic growth at fast growth rates, (3) to see how far the predictions of the Hartwell and Unger model apply to chemostat grown cells, and (4) to compare the results with those of the same strain in batch culture to see whether the quantitative relationships are similar.

RESULTS

Asymmetrical Age Distribution

The results from the bud scar analyses are presented in Table 1. Estimates of the daughter cycle time (D), the parent cycle time (P) and the duration of the budded phase (B) were calculated using, the dilution rate of the culture, the number of unbudded daughter cells, the number of unbudded parent cells, the number of budded cells, the total number of scars and the equations derived from the Hartwell and Unger model by Lord and Wheals (1980). The results are shown in Table 2 and the values of D, P and B plotted against population doubling time (τ) in Figure 1. The results are presented on a double logarithmic plot in order to accommodate the large number of points at the lower end of the scale. There are clearly two distinct phases for all three parameters, both of which are linearly related to τ (Table 3).

It was suggested (Lord & Wheals, 1980) that the age distribution for asymmetrically dividing cells became the same as that for symmetrically dividing cells at the maximum balanced growth rate, i.e. at μbmax, D=P=τ. If the lines of D and P at the slower growth rates are extrapolated D would equal P and τ at 170 minutes. This does not occur because at doubling times less than 200 minutes a second phase occurred in which the fraction of daughter cells remained constant at approximately 55%. The ratio of D:P is shown in Table 2 and the constancy of D clearly visible in Figure 2, where the ratio of D:P is plotted against the specific growth

Table 1. Numbers of budded and unbudded parent and daughter cells at different τ .

τ (min.)	μ (h^{-1})	Unbudded daughters	Budded daughters	Unbudded parents	Budded parents	Budded cells	Bud scars	Total cells
80	.521	239	357	97	363	720	918	1038
90	.460	220	342	86	376	718	961	1024
95	.438	264	332	54	414	746	1005	1064
107	.389	260	331	90	367	698	868	1049
114	.366	235	332	69	375	707	918	1011
128	.326	278	311	81	364	675	926	1034
136	.307	243	349	78	374	723	927	1044
146	.286	240	389	101	310	699	739	1040
167	.249	160	398	104	355	753	948	1017
174	.238	193	379	129	349	728	920	1050
196	.212	364	346	165	331	677	1075	1200
200	.208	200	342	97	381	723	886	1020
241	.172	466	259	225	256	515	958	1206
322	.129	463	224	117	209	433	825	1013
361	.115	555	137	229	180	317	870	1209
398	.104	536	89	203	179	268	798	1007
429	.097	589	73	198	166	239	893	1026
540	.077	613	81	184	143	224	673	1021
735	.057	1364	159	289	241	400	1177	2053
736	.056	700	71	224	126	197	752	1121

Table 2. Duration of cell cycle parameters D, P, B, P-B, D-B and the ratio of D:P at different τ. All values are expressed in minutes.

	Duration of parameter (min.)					
τ	D	P	B	P-B	D-B	D:P
80	88	72	60	12	28	1.36
90	100	81	70	11	30	1.22
95	111	81	73	8	38	1.27
107	120	95	80	15	40	1.30
114	129	100	87	13	42	1.28
128	149	109	93	16	56	1.32
136	153	120	104	16	49	1.31
146	157	135	109	22	48	1.53
167	173	160	134	13	39	1.22
174	182	168	132	36	50	1.27
196	223	171	127	44	96	1.43
200	214	186	155	31	59	1.13
241	279	207	126	81	153	1.51
322	432	234	170	64	262	2.11
361	467	274	136	138	331	1.90
398	537	287	140	147	397	1.65
429	625	273	131	142	494	1.82
540	781	356	165	191	616	2.12
735	1083	474	210	264	873	2.20
736	1189	420	184	236	1005	2.87

Table 3. Daughter cycle time (D), parent cycle time (P) and budded period (B) as a function of the population doubling time (τ).

The values were obtained by linear regression, the correlation coefficient being good in all cases. The fast growth rates included values faster than τ = 200 minutes: the slow growth rates included values slower than τ = 196 minutes. All values are expressed in minutes.

Period	Fast growth rates	Slow growth rates
D	$1.04\tau+9$ ($r^2 = 0.98$)	$1.71\tau-128$ ($r^2 = 0.99$)
P	$0.96\tau-8$ ($r^2 = 0.98$)	$0.50\tau+82$ ($r^2 = 0.97$)
B	$0.71\tau+6$ ($r^2 = 0.95$)	$0.11\tau+108$ ($r^2 = 0.61$)

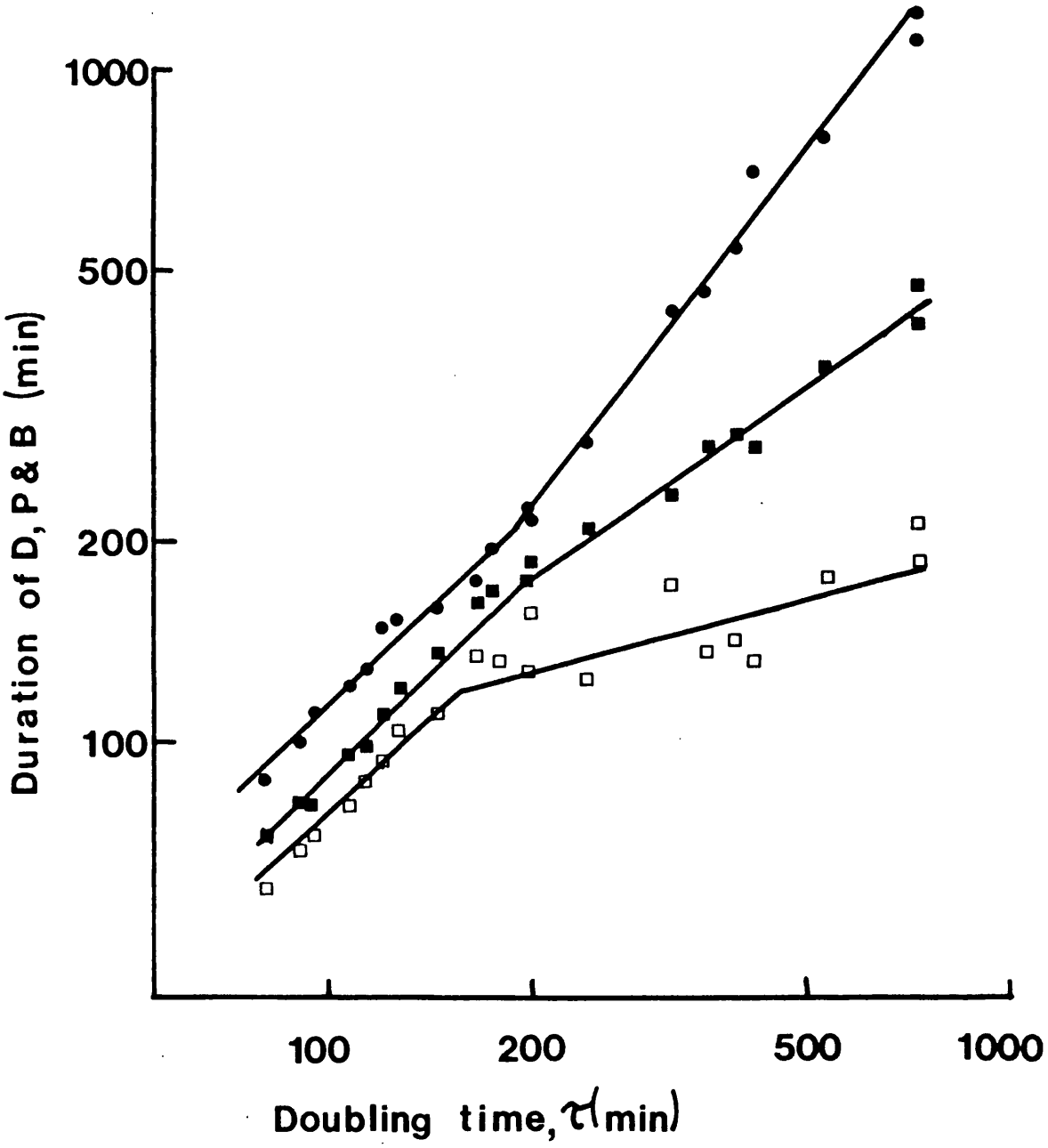


Fig 1. Daughter cycle time D•, parent cycle time P■, and budded period B□ as a function of the population doubling time τ. The scale is logarithmic on both axes.

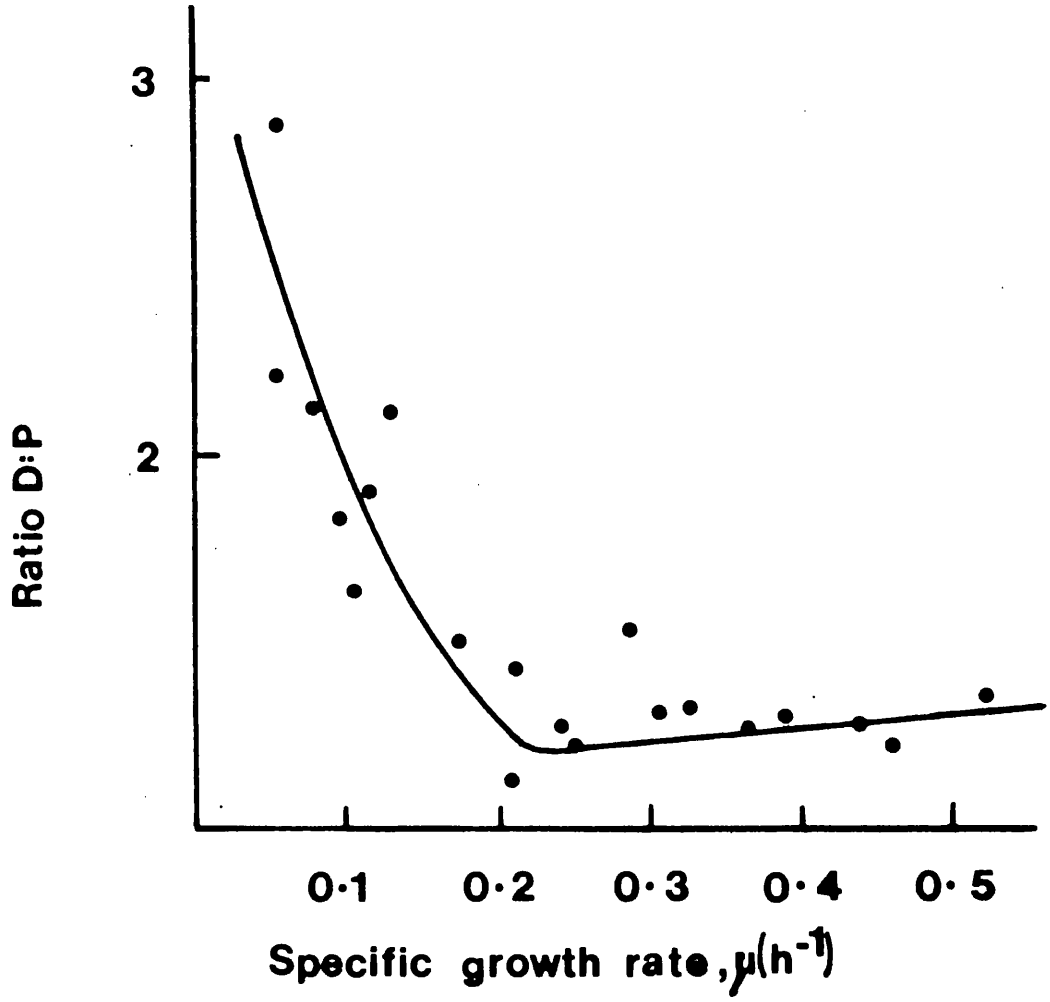


Fig 2. Ratio of daughter D to parent P cycle time time as a function of the specific growth rate μ.

rate (μ). As μ decreased from 0.2 to 0.05, the ratio of daughters to parents increased until at the slowest growth rates there are more than twice as many daughters as parents in the population. Bud emergence is dependent on start (Hartwell et al, 1974) and it has been suggested that start occurs at the birth of a parent (Lord & Wheals, 1980). A plot of P-B against τ would reveal whether the interval between these two events was constant or varied with the growth rate. Figure 1 indicates the P-B interval increased linearly with τ except at the fastest growth rate where it seemed to remain constant.

At doubling times less than 80 minutes small, stable clumps of yeast cells were found constituting only 1-2% of the population (see below).

Figure 3 compares the values of D, P and B found in chemostat culture with those found in batch culture (Lord & Wheals, 1980) over the same range of doubling times (70-250 minutes). Apart from the biphasic nature of the chemostat values the main differences from batch culture are a longer P and B and a shorter D period.

Genealogical Age Distribution

The method and theory of determining the genealogical age distribution have been described by Lord and Wheals (1980). The predicted values were calculated using the two estimates of P in Table 3. Figure 4 shows the predicted variation in the percentages of cells of different genealogical ages with τ. At doubling times

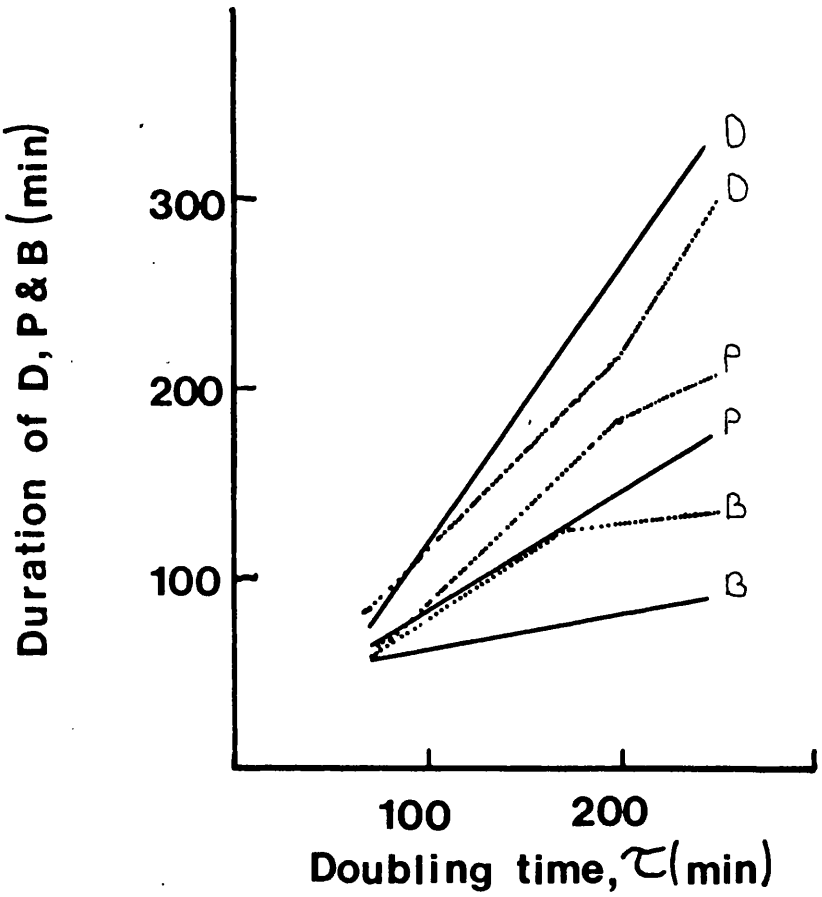


Fig 3. Daughter cycle time D, parent cycle time P and budded period B as a function of the population doubling time τ. Batch-grown cells —— and chemostat-grown cells ·····.

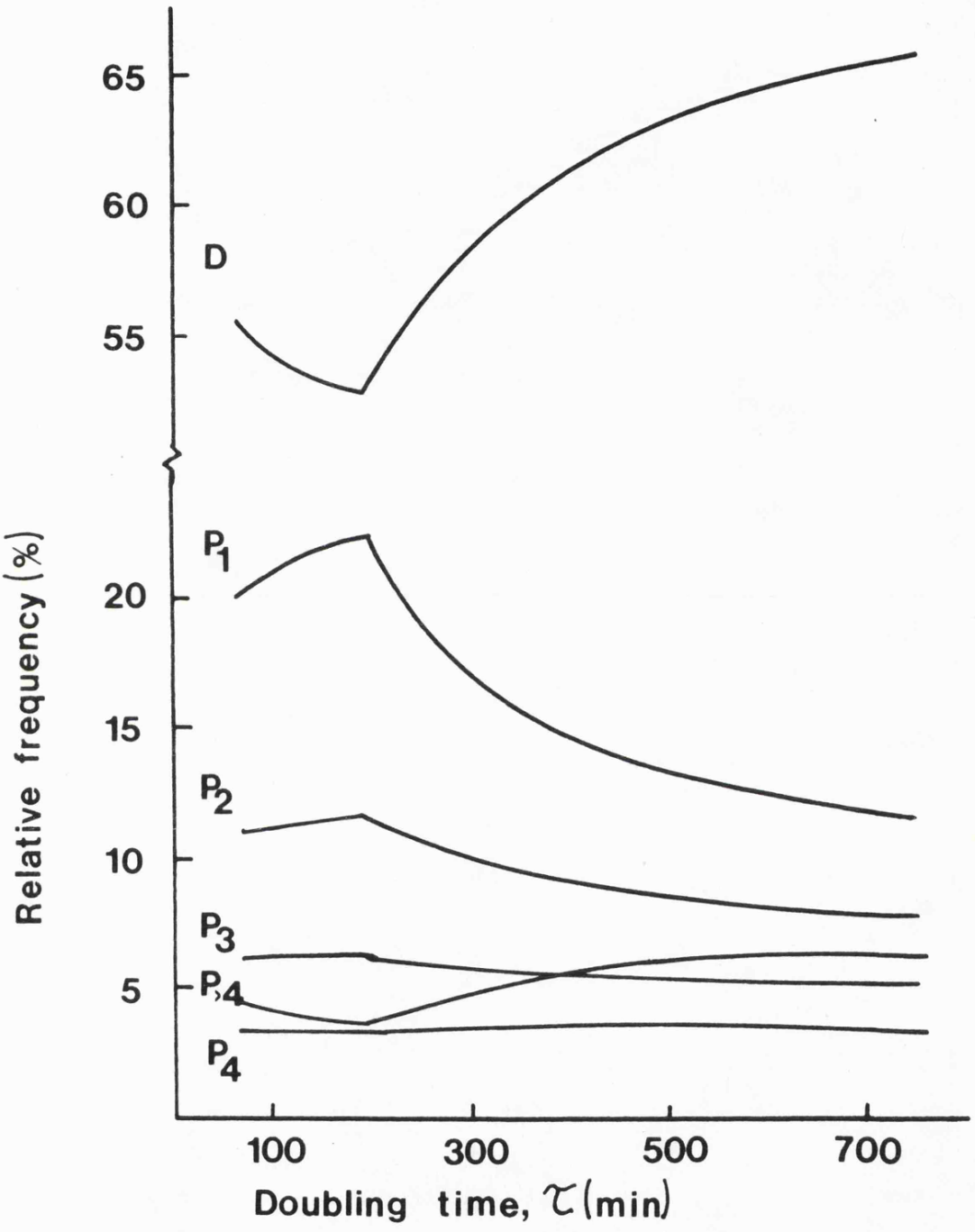


Fig4. Relative frequency of cells of different genealogical ages as a function of the population doubling time τ. The curves are calculated from Lord and Wheals (1980) and Table 3

Table 4. Percentages of cells at different genealogical ages at each τ.

τ (min.)	D	Percentage cells at genealogical age P_1	P_2	P_3	P_4	Pn 4
80	56.52	21.96	10.98	5.88	3.08	2.41
90	54.88	22.07	12.69	4.79	3.42	3.13
95	56.02	20.96	11.65	5.17	2.26	3.95
107	56.43	23.07	11.44	4.58	2.38	2.29
114	56.08	20.47	11.47	6.23	2.77	2.97
128	56.96	20.60	10.35	5.61	3.48	3.00
136	56.70	20.88	11.40	5.46	2.68	2.97
146	60.48	20.38	11.73	4.04	1.92	1.44
167	54.87	21.93	11.60	5.80	2.75	3.05
174	55.86	20.51	12.21	5.86	2.83	2.73
196	59.17	18.25	11.17	5.83	3.17	2.92
200	53.14	24.22	13.23	5.49	2.06	1.86
241	60.11	19.80	10.86	4.98	2.32	2.32
322	67.82	12.64	7.80	6.22	3.16	3.36
361	65.51	15.88	9.35	4.38	2.40	2.40
398	62.07	18.57	9.63	4.07	2.48	3.18
429	64.52	14.72	8.19	4.77	3.70	4.09
540	67.97	14.89	8.91	4.11	2.35	1.76
735	68.78	15.00	7.85	4.10	1.78	2.50
736	74.18	12.32	6.70	3.12	2.24	2.44

greater than 200 minutes the genealogical age distribution varied due to the pronounced asymmetrical cell division. At doubling times less than 200 minutes there was little predicted change in any of the different age classes, as expected from Figure 1. Experimental values of the percentage of cells at different ages is shown in Table 4 and the values for cells of age 1 and age 2 are plotted in Figure 5. The data give a good fit to the predicted values for the asymmetrical division model and clearly do not fit the predictions of a symmetrical division model where the percentage of P_1 and P_2 should remain constant at 25 and 12.5% respectively.

The data can be analysed by a "box and whiskers" plot (Figure 6). The plot illustrates the relationship of the observed to expected frequencies of cells of different genealogical ages over the range of growth rates used. As the relative frequency of cells declines with genealogical age, analysis is normalised by taking square roots of both observed (O) and expected (E) values in order to make the differences of comparable magnitude. The difference between the square roots of the observed and expected frequencies at each of the twenty growth rates is presented in the form of a box enclosing the central 50% of the data points (the median is indicated by a cross bar), and whiskers extend to the extreme values (a difference of $\pm$ 0.5 on the ordinate indicate a departure from the expected value of $\pm$ 16 when the number counted was 250). The data does not follow the general trend predicted from the

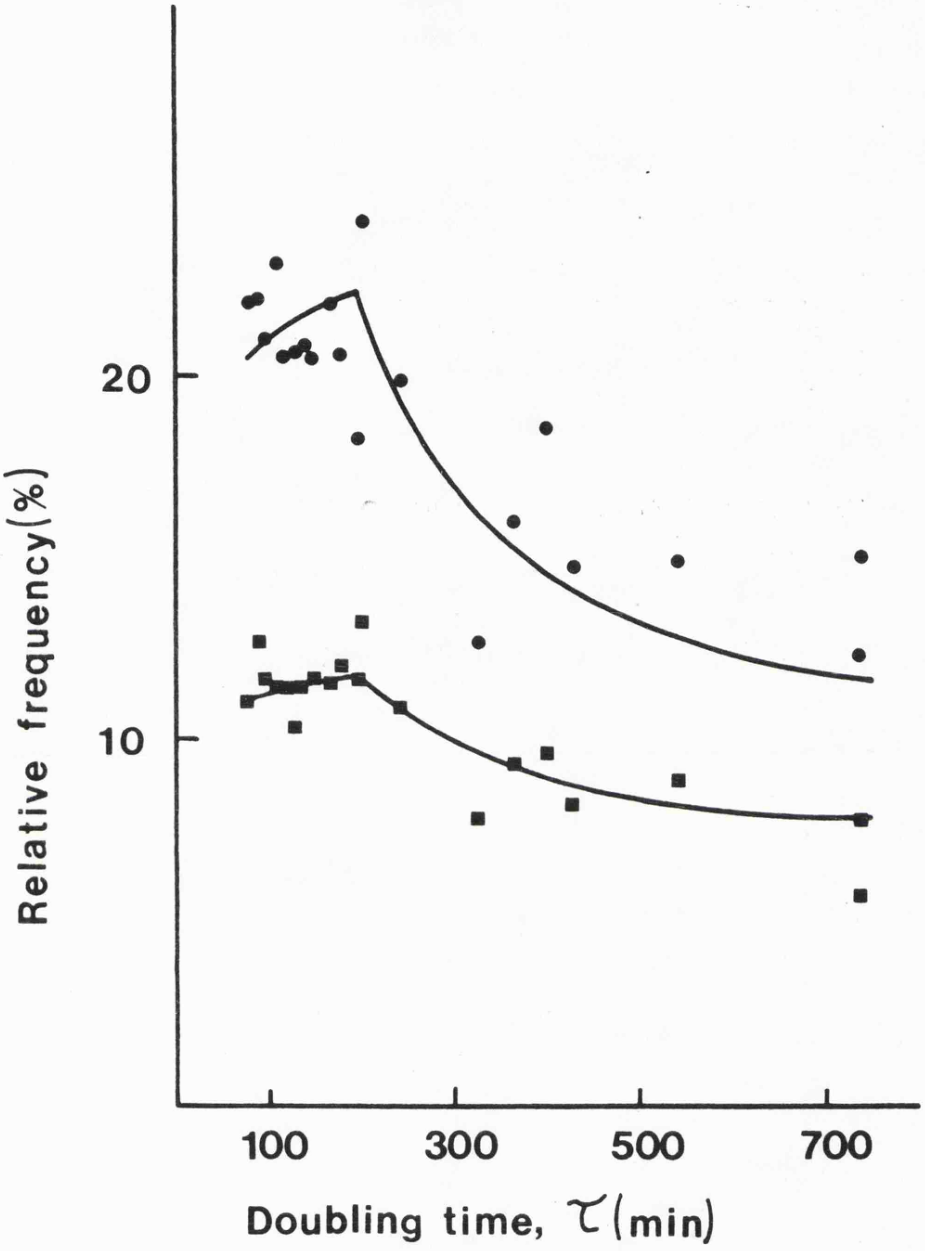


Fig 5. Relative frequency of parent cells of age 1 • and age 2 ▪ as a function of the population doubling time τ. Points are experimental values; curves are from Fig. 4.

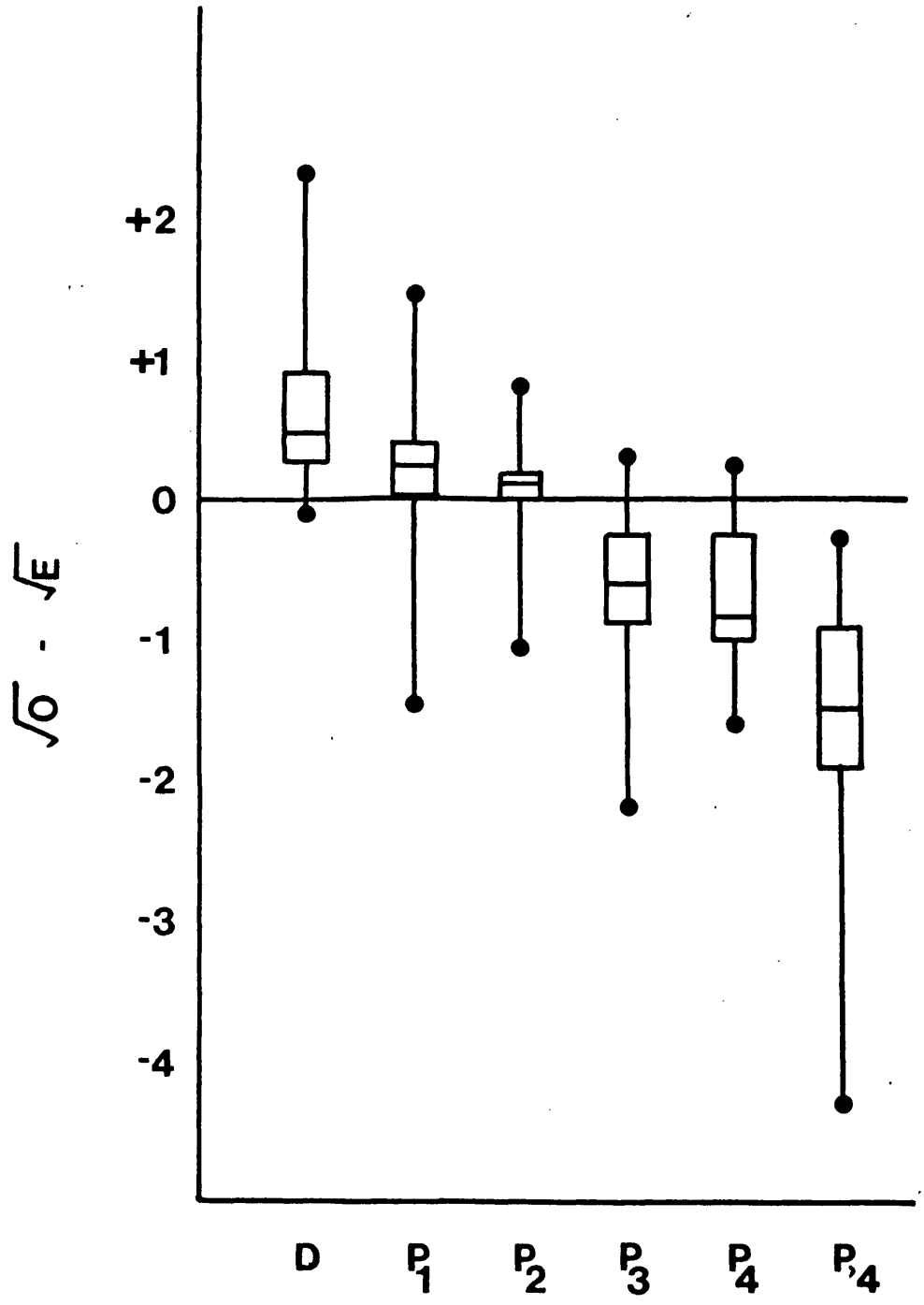


Fig 6. Relationship of observed to expected frequencies of cells of different genealogical ages over the range of growth rates.

model in that there is an excess of daughters and fewer cells of genealogical age 3 and over. This was particularly noticeable at the slowest growth rate.

Cell Volume, Dry Weight and Density

Median cell volume was constant at the slower growth rates 736-300 minutes but increased at doubling times faster than 300 minutes to give a two fold increase at the fastest rates (Figure 7a). The dry weight and number of cells per unit volume was measured and the dry weight per cell calculated (Figure 7b). The shape of the curve is similar to that of the median cell volume indicating that it is not an unspecified osmotic effect, but a real effect of macromolecular synthesis. The dry weight per cell was divided by the median cell volume to give median cell density (Figure 7c). This revealed a biphasic relationship of density to τ, density decreased at doubling times from 736-150 and then sharply increased at the fastest growth rates. All values are shown in Table 5.

Position of the Bud Scar

The position of the bud scars on cells of different genealogical ages at different doubling times, expressed as a percentage of cells with buds at the same pole is given in Table 6, and the total percentage is plotted against doubling time in Figure 8. At the fastest growth rates virtually all cells had their bud scars at the same pole in a precisely ordered sequence, but as the doubling time increased so did the fraction of cells with bud scars at both poles,

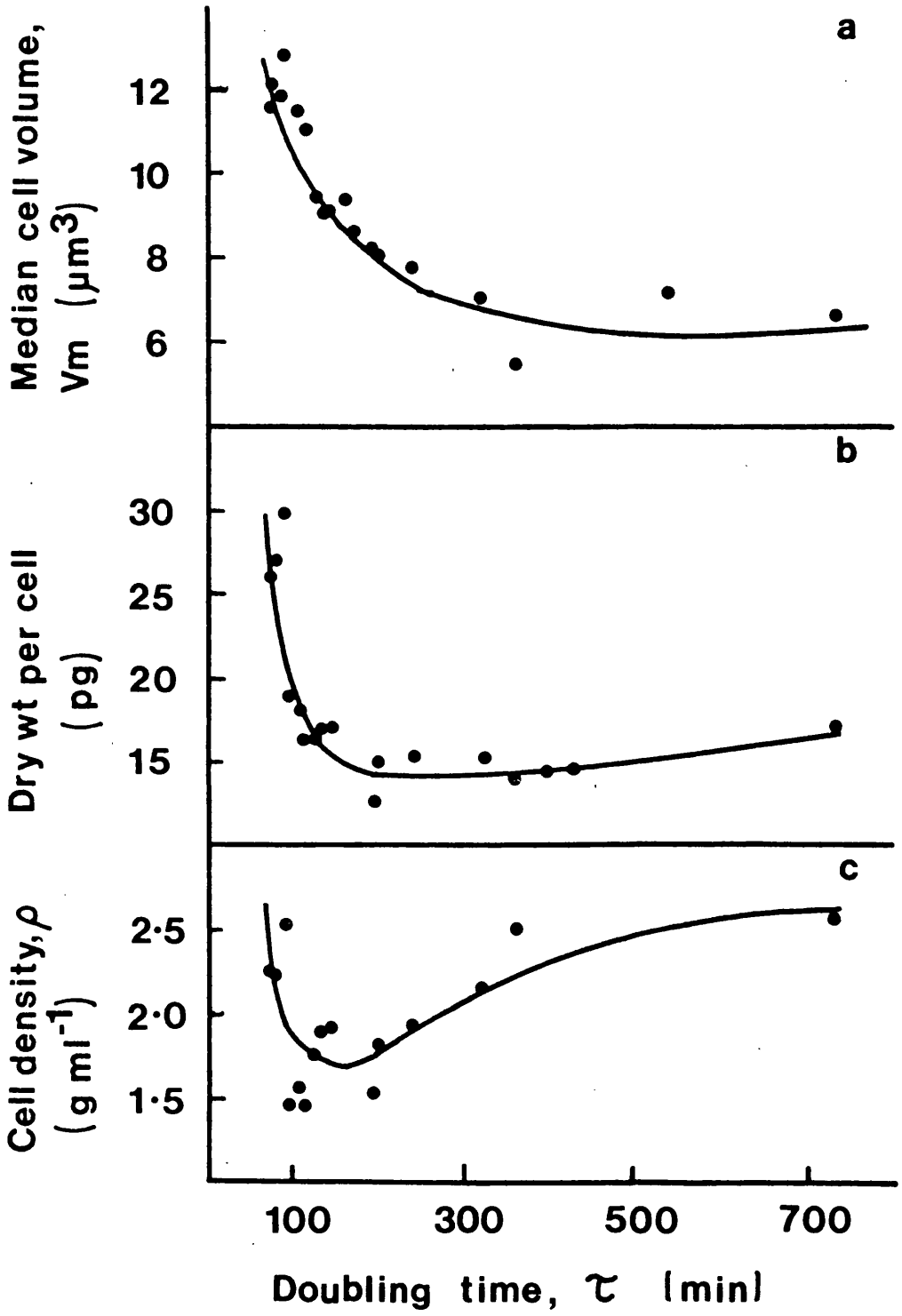


Fig7. a Median cell volume, b Dry weight per cell and c Average cell density as a function of the population doubling time τ. The curves a and b were fitted by eye and used to construct curve c.

Table 5. Cell number, dry weight per cell, volume and cell density at each τ .

τ (min.)	Cell number ($x10^8$/ml)	Dry wt/cell ($gx10^{-12}$)	Volume (μm^3)	Cell density (gml^{-1})
75	0.83	26.15	11.61	2.25
80	0.84	26.86	12.15	2.21
90	1.13	29.8	11.83	2.52
95	1.99	18.77	12.81	1.47
107	2.49	18.02	11.54	1.56
114	2.67	16.38	11.12	1.47
128	3.03	16.47	9.39	1.75
136	3.82	16.96	8.97	1.89
146	4.12	17.09	8.94	1.91
167	5.26	NM	9.32	-
174	6.97	NM	8.55	-
196	7.04	12.60	8.25	1.53
200	8.07	14.78	8.11	1.82
241	8.62	15.19	7.81	1.94
322	8.32	15.20	7.05	2.15
361	8.65	13.82	5.49	2.5
398	8.56	14.29	NM	-
429	9.11	14.60	NM	-
540	8.64	NM	7.15	-
735	8.80	17.04	6.62	2.57
736	8.45	NM	NM	-

Table 6. Percentages of cells with buds at the same pole for each genealogical age at each growth rate.

τ (min.)	Cells with buds at the same pole (%) P_1	P_2	P_3	P_4	Pn 4	Total
80	99.5	99.1	95	100	100	98.7
90	99.5	99.2	100	100	96.9	99.3
95	98.5	99.2	100	100	100	99.1
107	100	98.3	97.9	96	95.8	98.8
114	99.4	98.3	100	100	100	99.3
136	99.5	98.3	94.7	100	93.6	98.1
146	99.4	97.5	95.2	85	100	97.5
167	98.9	90.7	84.8	82.1	61.3	90.4
174	94.0	95.2	76.7	82.8	60.7	88.4
200	94.2	80.7	69.6	61.9	94.7	85.0
241	80.2	74.1	73.9	66.7	44.4	74.6
322	70.5	63.3	36.5	31.3	14.7	50.7
361	81.8	67.2	73.1	68.4	53.9	72.8
540	74.0	63.2	41.1	35.3	11.6	52.8
735	48.3	33.0	19.6	10.0	3.6	29.3

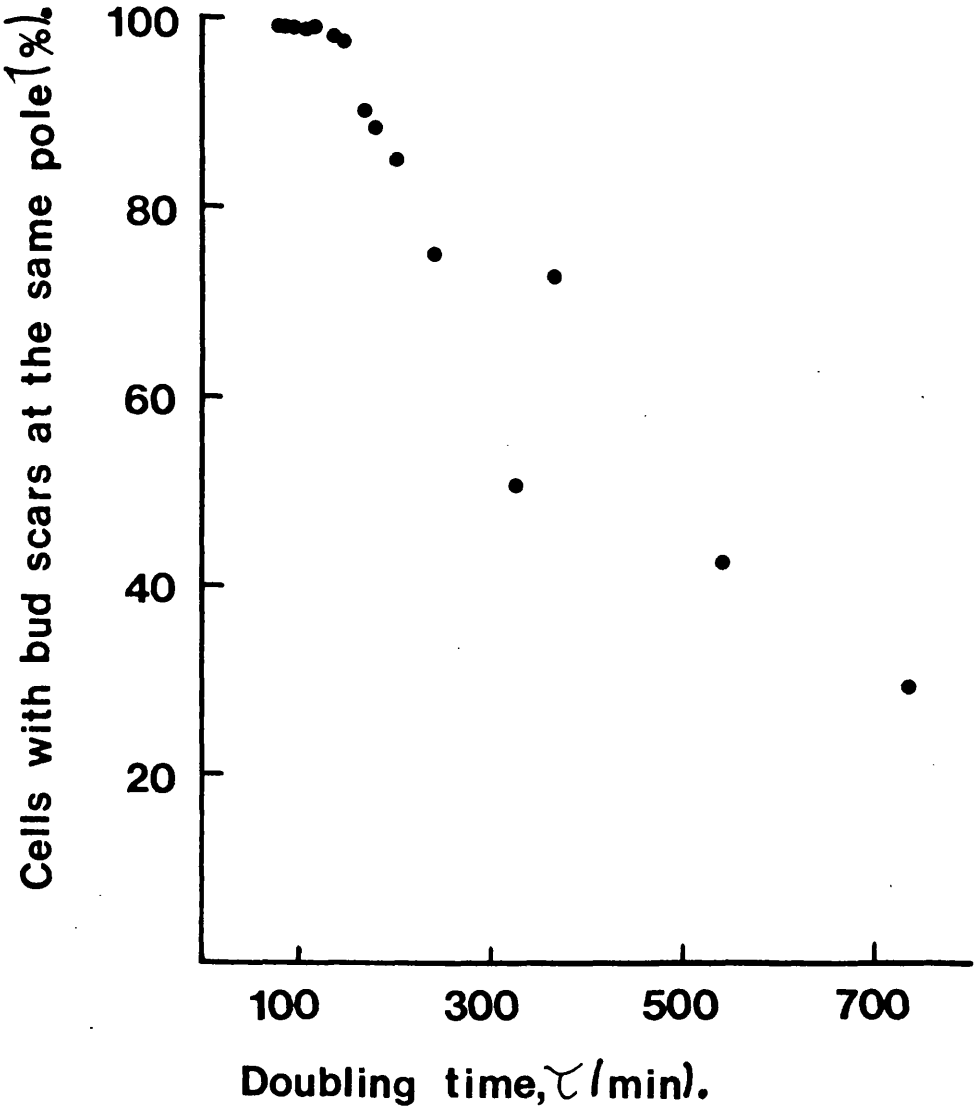


Fig 8. Percentage of cells with a contiguous array of bud scars as a function of the population doubling time τ.

or with gaps in the sequence of scars. It can be deduced that the gaps in the sequence are not filled in during subsequent cycles since the fraction of cells with disordered sequences always increased with increasing cell age (Table 6).

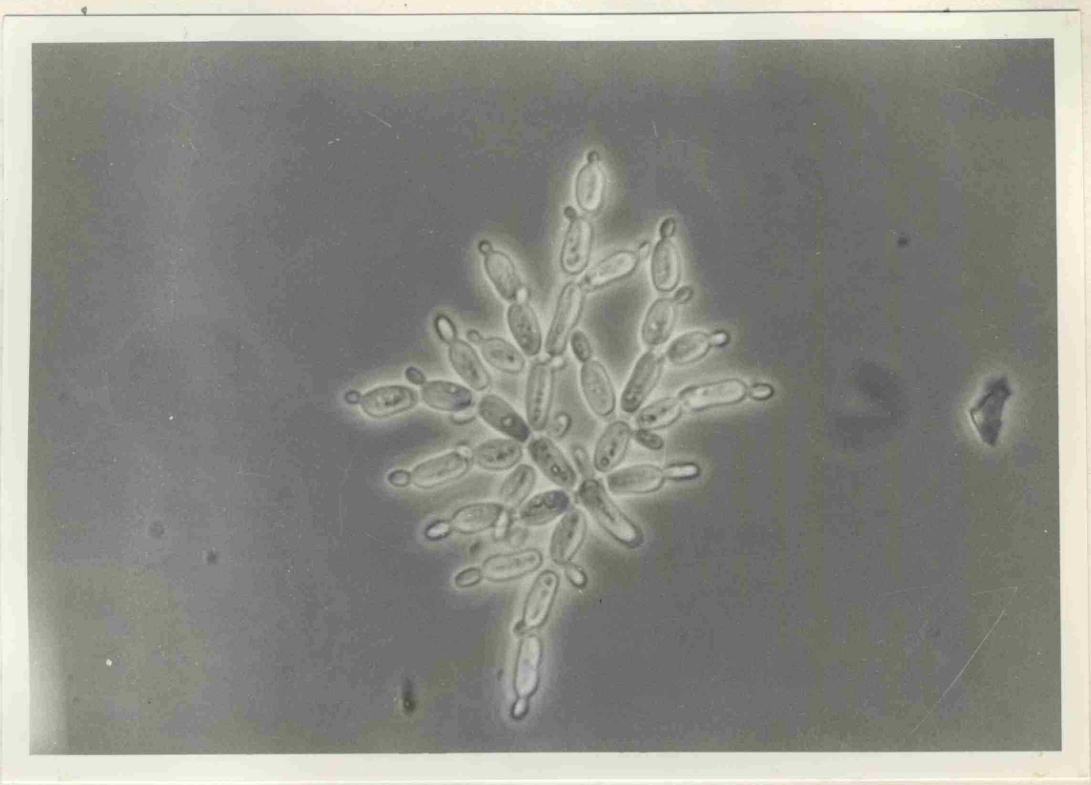
Filamentous Forms

At doubling times less than 80 minutes small stable clumps of yeast cells were found (Figure 9a), the number of individual cells in these clumps constituting 1-2% of the population. The individual cells could not be separated from each other by mild sonication; routinely performed on all chemostat samples. The clumps could be broken up into separate cells by gentle digestion with Zymolyase 5000 at concentrations that did not lyse the cells. This technique has been used previously to demonstrate that cells can be artificially separated once cytokinesis has occurred (Hartwell, 1971). The tracing (Figure 9b) shows that there are 16 daughters (age 0) and 16 parents of different genealogical ages, eight of age 1, four of age 2, two of age 3, and one each of ages 4 and 5. There is a high degree of synchrony with all but three cells having visible small buds. Bud scar analysis showed that asymmetrical division was occurring for the bulk of the population (single cells), however symmetry is evident for the filaments. Filaments of four to eight cells usually showed signs of synchrony but as the number of cells increased so did the lack of synchrony. When the filaments were transferred to agar-slide culture they remained stable but budded off normal single cells. The enzyme digestion and sonication experiment strongly suggested that the cells had undergone cytokinesis

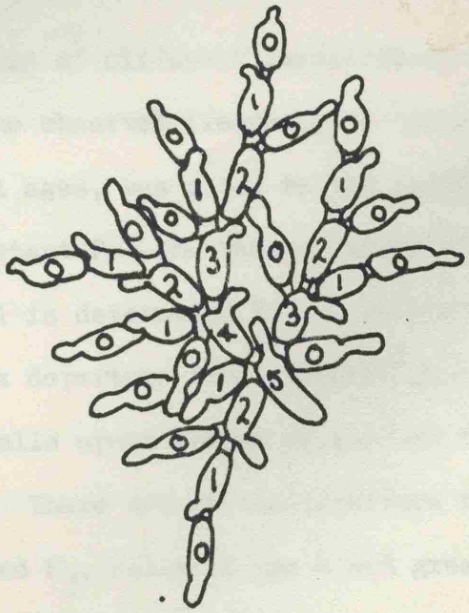
and probably primary septum formation (Cabib, 1975) but had failed to complete cell separation.

Figure 9. (a) Phase-contrast micrograph of filament-forming yeast cells.
(b) Drawing of (a) with genealogical age of the cells marked. There are 16 daughters (age 0), eight parents of age 1, four of age 2, two of age 3, and one each of age 4 and 5. Twenty-nine of the 32 cells have small buds.

a.



b.



DISCUSSION

Cell Cycle Parameters in the Chemostat

In batch culture experiments it has been shown that the Hartwell and Unger model (1977) of asymmetrical cell division provided a quantitative explanation of the experimental estimates of D, P and B, and the observed values of F_D and $F_{P(n)}$ (Lord & Wheals, 1980). It was possible to test independently a number of assumptions of the model and demonstrate that they were valid. The same tests were applied to the present data and the same general conclusions emerge. In populations of cells growing in a chemostat under balanced steady-state exponential growth conditions it can be concluded that at any one growth rate,

(i) division was asymmetrical (Figure 1),

(ii) $D > P > B$ (Figure 1)

(iii) P was constant for cells of different genealogical age. Figure 6 shows that the observed frequency of cells of different genealogical ages, was close to the predicted value, assuming a constant P. The rate of entry of parents into age $\underline{n} + 1$ is determined by the rate of exit of parents of age $\underline{n}$, a departure from expected values could occur only if cells spent longer or shorter times in each parent cycle. There was little departure for cells of age P_1, P_2 and P_3, cells of age 4 and greater could have longer or shorter cycle times, however this may not be the case. The Hartwell and Unger model assumes immortality of cells, but if there was an increasing

probability of death with increasing genealogical age this would decrease F_P and increase F_D above its expected value, as was observed. It should also be noted that at the higher genealogical ages very few cells were counted compared to lower ages.

(iv) B was the same for daughters and parents of all genealogical ages. This was tested by calculating the fraction of budded parents at each age for each growth rate (F_{PBn}) and subtracting this value from the mean value ($\bar{F}_{PB}$) of the five age groups at that growth rate (Table 7). There was no evidence that the fraction changes significantly at each genealogical age. Hence it can be concluded that the budded phase for parents of different genealogical ages was constant.

(v) The genealogical age distribution was as predicted by the model (Figure 4).

This analysis revealed a clear biphasic linear relationship of D, P and B to τ, the transition point being at about 200 minutes in all cases. The monophasic linear relationship found in batch culture studies was only extended to doubling times as slow as 250 minutes and thus may only have been relevant to faster growth rates (Lord & Wheals, 1980). Average cell density (ρ) also changes at a doubling time of about 200 minutes, reaching a minimum at this doubling time.

The transition point for D, P and B corresponds almost exactly to that found by Beck and von Meyenberg (1968) in their analysis of

Table 7. Median value of $(\bar{F}_{B} - F_{Bn})$ for each parental age group +

Genealogical age	Median value of $(\bar{F}_{PB} - F_{PBn})$
1	0.0353
2	0.0005
3	0.0022
4	-0.0205
4	-0.0697

+ F_{PBn}, Number of budded cells/total number of cells for parental age group n; $\bar{F}_{B}$, mean value of F_{Bn} when n = 1, 2, 3, 4 and 4.

the level of certain enzymes of Saccharomyces cerevisiae in glucose-limited chemostat culture at 30°C. At doubling times less than 200 minutes they concluded that the cells were growing by "aerobic fermentation" while at slower growth rates "oxidative metabolism" was occurring.

Comparison of Chemostat and Batch Culture

Figure 3 compares the values of D, P and B found in chemostat culture with those found in batch culture over the same range of doubling times (70-250 minutes). The values for batch-grown cells were redrawn from Lord & Wheals (1980). Apart from the biphasic nature of the chemostat values, the main differences from batch culture are longer P and B periods and a shorter D period.

The relationship of D, P and τ in equation

$$e^{-\alpha D} + e^{-\alpha P} = 1 \quad \text{where} \quad \alpha = \ln 2/\tau$$

reveals that once two of the parameters are set, the third can be calculated. Biologically under balanced growth conditions when mass doubling time equals population doubling time, with τ set, it is P that determines D. This is because the daughter cycle time is determined by the period needed for a daughter to grow from birth size to critical size, at which parents are born (Johnston et al, 1977). The birth size of the daughter is determined in turn by the time during which mass is synthesized in the parent cycle and its partitioning into the bud during the B period. Since B is a part of P, an elongation of B will also elongate P. The extended attachment of a bud to a parent will result in a larger daughter

at birth, which will thus have a correspondingly shorter cycle time. The elongation of the B period and the interdependence of D, P, B and τ seem to be the principal reasons for the altered relationship seen in chemostat culture, where the B period can be as much as 50 minutes longer.

The cause of the elongation is unknown, but it could be related to relief from catabolite repression. During exponential growth in batch culture the carbon substrate is present in excess while in chemostat culture the substrate is certainly growth-limiting at doubling times greater than 100 minutes (Beck & von Meyenberg, 1968). Such an explanation predicts that at the fastest growth rates, when the media in chemostat and batch culture are identical, each parameter should be identical in the two conditions. At τ = 80 minutes each value in batch culture is within 6 minutes of the value in chemostat culture.

The D and P curves for batch culture converge to equality at τ = 65 minutes. Although unattainable, it was predicted (Lord & Wheals, 1980) that at such a growth rate symmetrical division would occur. No such expectation was predicted from extrapolation of the chemostat data, yet symmetrical division was seen amongst a small percentage of cells when they produced filaments at $\tau <$ 80 minutes. The symmetry was revealed by the identity of size and the synchrony of bud emergence of parents and daughters (see below). This observation again points to a similarity of behaviour of chemostat- and batch-grown cells at the fastest growth rates.

Cell volume differences were also seen between chemostat and batch cultures. Saccharomyces cerevisiae cells grown in either glucose-limited or ammonium-limited chemostats are larger at faster-growth rates (McMurrough & Rose, 1967; Mor & Fiechter, 1968), as has been found here where there is a two fold difference in median cell volume over the growth rate studied (Figure 7). More interestingly, the largest cells are still smaller than the smallest cells of the same strain grown at fast growth rates in batch culture (Lord & Wheals, 1980), in contrast to the identical volumes found in similar work by Johnston et al (1979). The median cell volumes differ because the initial size for traverse of start is dependent on growth rate (Lord & Wheals, 1980). A similar nutrient-modulated size control over division has been seen in S. pombe (Fantes & Nurse, 1977).

The conclusions from a comparison of chemostat- and batch-grown cells is clear. The biology of cells growing at the same growth rate can be quite different and caution should be exercised in extrapolating from one set of conditions to another.

Position of the Bud Scar

Starting from the birth scar, buds on haploid cells are formed in highly ordered sequences as rings, rows or spirals (Streiblova, 1970; Freifelder, 1960). Cytoplasmic microtubules extend from the spindle pole body (SPB), embedded in the nuclear membrane, to the base of, and sometimes into, the emerging bud. After division, the SPB is at the opposite pole to both the birth

scar in the daughter cell and the last bud scar in the parent cell, so that if the SPB controls the location of the new bud it will have to re-orientate precisely at each cycle (Lord & Wheals, 1980). One possible source of spatial information could be the microtubules extending in from the last bud scar. At doubling times less than 136 minutes over 98% of the population showed this precise control but as the growth rate fell so did the degree of precision (Figure 8). The irregularities could appear at any genealogical age with the result that the oldest cells in the population had highest probability of showing an irregular pattern.

Filament Formation

The stable clumps of filaments observed at the fastest growth rates were of great interest (Figure 9). This phenomenon seems to be distinct from the three other known causes of filament formation in yeasts. First, chain formation is well known to occur, particularly among brewing strains of Saccharomyces cerevisiae. However, it is found in all cells of the population and at all growth rates. Furthermore, the chains tend to break up during the stationary phase of the growth cycle, in contrast to the stable structures revealed here (which can nevertheless bud off individual cells). Secondly, dimorphism has been frequently observed among the yeast-like fungi (Stewart & Rogers, 1978) although not with Saccharomyces cerevisiae. More importantly, observations on Mucor rouxii grown in chemostat culture (Bartnicki-Garcia, 1968) showed that slow growth rates favoured the filamentous form, and fast growth rates the yeast-like form, the opposite of what was seen here. Thirdly,

the phenomenon does not seem to be pseudomycelial growth, since there was no marked elongation of the bud, characteristic of this type of development. The structures described are perhaps best regarded as true filamentous mycelia with complete septation, the individual 'compartments' being more cylindrical than the normal prolate spheroids of the individual cells.

The filaments do not seem to be formed by the same mechanism that causes secondary bud formation, since in filaments cell separation is abolished, whereas in secondary bud formation it is merely delayed. Although cytokinesis and cell separation are triggered by the sequential pathway of the cell division cycle, they are not an integral part of the cycle and a further cycle can be initiated before they are complete. This is in agreement with the results of Hartwell (1971) using cell division cycle mutants blocked in cytokinesis and cell separation.

It can be seen that all cells had their buds at the opposite pole to that of the birth scar, in contrast to the free individual cells (see above). This filamentous mode of growth, away from the pole at which a cell was born, leads to an interesting comparison of the cell cycle of the normally unicellular yeast Saccharomyces cerevisiae with the duplication cycle for filamentous moulds (Fiddy & Trinci, 1976; Trinci, 1978).

The duplication cycle has three principal features which can be summarised as: (1) the apical compartment is reduced to half its

length by septation; (2) the apical compartment increases in length at a linear rate; (3) critical nuclear concentration triggers a round of mitosis and septation to be completed after a doubling in size of the apical compartment. The last completed cycle of Figure 9(a) budded off cells age 0 from cells of variable age. The mean length of age 0 cells is 93% that of their parents, indicating approximately symmetrical septation and feature (1). An examination of the chain of cells age 0 to age 5 shows them to be of comparable size indicating that the filament has extended linearly during six generations (feature (2)). The trigger for initiation of events leading to mitosis and septation is traverse of start (Hartwell et al, 1974), which is dependent, under balanced growth conditions on the attainment of a ploidy-related critical cell size, i.e. nuclear concentration (Hartwell & Unger, 1977; Lorincz & Carter, 1979). This event occurs at or near birth in cells growing at fast rates (Lord & Wheals, 1980). In the present experiments completion of the cycle occurred at cytokinesis when the daughter cells had reached the same size as their parents, that is, after a doubling in cell size, demonstrating feature (3).

In addition, a number of other features of septate mycelial growth (Trinci, 1979) are shown by these filaments. First, growth only occurs at the tips of a mycelium. The marginal increase in size of older cells compared with younger cells (Figure 7a) reinforces the observation (Bayne-Jones & Adolph, 1932) that growth of budding yeast largely occurs by growth of the bud

(= tip of the mycelium). Secondly, there is an exponential increase in the number of tips, shown in Figure 7b by the perfectly symmetrical genealogical age distribution with 32 cells producing 32 tips (29 having just budded, 3 still to bud). Thirdly, growth in older compartments occurs from immediately behind the septa, as shown in Figure 7a by the position of the growing bud in the parent cells (age> 1) being always behind the septa of a previous budding cycle.

The filamentous mode of development of Saccharomyces cerevisiae parallels the duplication cycle and other features of filamentous mould growth, lending weight to the view that differences in yeast-like and mycelial growth may often be of degree rather than kind.

From the results of this study, it is proposed that the quantitative predictions of the Hartwell and Unger model can be applied to glucose-limited chemostat culture as well as to batch culture. Although differences do exist between cells grown in batch and chemostat culture which affect the subsequent biology of the cell, these differences are quantitative not qualitative. The biphasic nature of Figure 1 and the presence of the filaments reinforces the proposal that aspects of the yeast cell cycle should be analysed over as wide a range of growth rates as possible.

CHAPTER FOUR

5'-dTMP Work

INTRODUCTION

thy⁻ Mutants in Bacteria

Understanding of the control and timing of cell cycle events in bacteria has been greatly enhanced by the technique of limiting the amount of exogenous thymine to thymine requiring mutants. This effectively slows down the rate of replication without affecting the growth rate.

In 1968 Cooper and Helmstetter showed that the transit time of replication forks (C) in E.coli was more or less constant in cultures with doubling times ranging from 70-20 minutes and that cell division followed at a constant time approximately 25 minutes later. This interval was designated D and Helmstetter (1968) suggested that the termination of rounds of replication provided a signal which initiated cell division. Therefore when the cell mass per chromosome origin reaches the critical value (Donachie, 1968) an increase in the rate of DNA synthesis is achieved solely by changing the frequency of initiation of rounds of replication.

The rates of DNA chain elongation and growth rate are not coupled since the growth rate can be altered without affecting the transit time of the replication fork. Pritchard and his co-workers considered the idea that it might be possible to alter the replication time without affecting the growth rate, and to find out what events were coupled directly or indirectly to DNA replication.

By reducing the thymine concentration in the growth medium of thymine auxotrophs it was possible to increase the chromosome replication time by a factor of 2.5 without significantly affecting the growth rate (Pritchard & Zaritsky, 1971). The longer C period was associated with a shorter D period (Pritchard, 1974). C was increasing at the expense of D. Although termination of replication was usually a prerequisite for cell division other factors were influencing the timing of division.

Average cell mass and volume are related to the thymine concentration; the lower the concentration, the higher the mass/volume (Zaritsky & Pritchard, 1973). Meacock, Pritchard and Roberts (1978) and Zaritsky and Woldringh (1978) proposed that the increase in volume was due to an increase in width with a slight decrease in length, while Begg and Donachie (1978) proposed that the increase was in length without a change in width. Thymine limitation of fast growing thy$^-$ strains resulted in the production of unusual morphologies apparently due to an unbalanced synthesis of wall components (Meacock et al, 1978). These mutants can be used in analysing the integration of cell wall growth with division, although a clear cut picture has not yet appeared.

Velocity-jump, or step-up experiments (where thy$^-$ cells are shifted from a low thymine concentration to a high concentration that supports a faster replication rate), have confirmed that, initiation mass is constant and independent of the thymine concentration at one growth rate, rounds of replication are initiated at a

unique site, and replication is bidirectional. They have also provided information on gene dosage and plasmid behaviour (Pritchard, 1974).

thy⁻ mutants have also been used in investigating DNA synthesis at the molecular level; in determing the numbers and rates of formation of Okazaki fragments (Brewin, 1977; Brewin & Cairns, 1977; Tomana & Okazaki, 1978), and in showing functional compartmentation of nucleotide pools at the replication fork (Pato, 1979).

thy⁻ cells in bacteria have proved to be invaluable in studies on DNA synthesis and the cell cycle, and the technique of increasing the replication time of the chromosome, a powerful tool for understanding the control and timing of cellular events.

5'-dTMP Mutants in Yeast

Saccharomyces cerevisiae is not permeable to either thymine or thymidine (Grenson, 1969; Brendel & Langjahar, 1974; Wickner, 1974), and has no detectable thymidine kinase activity (Grivell & Jackson, 1968), therefore neither specific labelling of DNA nor manipulation of the rate of DNA replication is possible with these compounds. Deoxythymidine monophosphate is converted from deoxyuridine monophosphate and after further phosphorylation is incorporated into DNA (Figure 10).

In 1968 Jannsen reported DNA specific labelling with deoxythymidine-5'-monophosphate (5'-dTMP) (Jannsen et al, 1968; 1973;

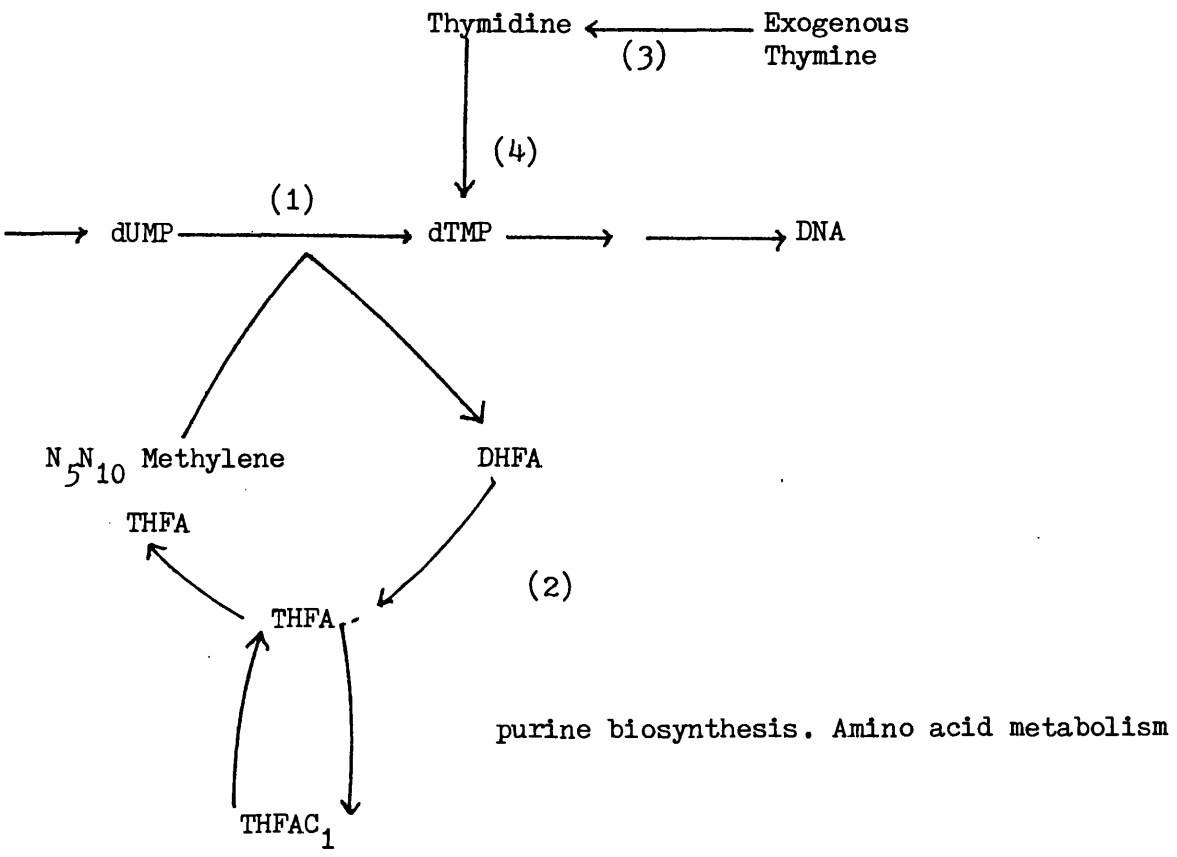


Figure 10. Folic acid metabolism and its relation to thymidylate biosynthesis in yeast.

Reactions (3) and (4) exist in bacteria but not yeast.

1 Thymidylate Synthetase
2 DHFA Reductase
3 Thymidine Phosphorylase
4 Thymidine Kinase

dTMP, deoxythymidine monophosphate
dUMP, deoxyuridine monophosphate
DHFA, dihydrofolic acid
THFA, tetrahydrofolic acid
C_1, carbon transfer

Jannsen, 1970), and Brendel and Haynes (1972) showed that the label was associated with both nuclear and mitochondrial DNA and that uptake was due to a single recessive gene. Laskowski and Lehmann-Brauns (1973) isolated further mutants from this strain caused by either one of three recessive genes linked to one another. Similar mutants were isolated by Wickner (1974) designated tup and controlled by four genes. Little and Haynes (1973) and Brendel (1976) selected four mutants with a more efficient uptake designated tum and controlled by two genes. It seems that a minimum of 4-9 genes confer permeability to 5'-dTMP.

Although the labelling is specific for DNA (Brendel & Haynes, 1973), only a small amount of the thymidine residues in DNA were derived from exogenous dTMP the rest being supplied by the normal pathway, conversion of dUMP to dTMP by thymidylate synthetase.

In bacteria thymidylate synthetase may be inhibited by folic acid antagonists such as aminopterin (APT) which impairs the activity of dihydrofolic acid reductase (O'Donovan & Neuhard, 1970). This will prevent dihydrofolic acid (DHFA) being reduced to tetrahydrofolic acid (THFA) and prevent thymidylate biosynthesis (Figure 10). All of the dTMP necessary for growth must then be obtained from external sources.

This was the rationale used by Laskowski and Lehmann-Brauns (1973). Total inhibition was not possible by APT alone, but if Sulfanilamide (SAA) was also added, which prevented any de novo

synthesis of DHFA, inhibition was complete. When nutrient medium containing these drugs was enriched with dTMP and adenine, growth was not possible. However, spontaneous mutants occurred that could grow. The technique was also employed by Fäth and Brendel (1974) who were able to generate DNA which totally derived its thymine contents from the external growth medium, and showed that 5'-dTMP passes through the cell walls and membranes to the site of DNA synthesis without being degraded, and that the amount of incorporation per unit time was dependent upon the concentration offered.

Brendel and Fäth (1974) isolated mutants auxotrophic and conditionally auxotrophic for 5'-dTMP, in the absence of APT and SAA, tmp mutants. All were respiratory deficient.

Two classes of mutants with a requirement for 5'-dTMP will be found; those with a simple requirement for 5'-dTMP defective in thymidylate synthetase, and those defective in folate metabolism which also require adenine, histidine and methionine. Little and Haynes (1979) have isolated and termed these second type, fol mutants. The tmp3 mutants of Luzzati (1975) fall into this class. They also selected for respiratory grande tmp mutants (Barclay & Little, 1978).

tmp mutants exhibit "thymineless death" (TLD) if 5'-dTMP is removed from the growth medium. After a lag of one doubling time cells lose viability exponentially arresting as doublets, which is typical of yeast in which DNA synthesis has been inhibited (Slater,

1973), or cdc mutants sensitive for DNA elongation at the restrictive temperature (Hartwell, 1973). Cell mass increased six fold and RNA and protein synthesis continues at a reduced rate.

Using gradient plates Brendel (1976) isolated thymidylate low requiring tlr strains (4 µg/ml 5'-dTMP) of tmp mutants.

The tmp1 gene is allelic to cdc21 (Game, 1976) and thymidylate synthetase activity is missing in in vitro extracts of these strains. The parental tup strains have similar enzyme activity to the wild type. tmp1 has been shown to be the structural gene for thymidylate synthetase (Bisson & Thorner, 1977).

Thymidylate starvation, as well as causing cell death, induces point mutations in the mitochondrial genome. Sub-optimal concentrations also induced petites (Barclay & Little, 1978). Similar observations have been made with cdc21 (Newlon et al, 1979).

High concentrations of thymidine also cause cell death "thymidylate excess death" (TED), and arrest with the morphology of cells undergoing TLD (Barclay & Little, 1978) - low requiring strains are particularly sensitive. The effects are both cytostatic and cytotoxic, 90% of cells losing their colony forming ability within one hour. RNA and protein synthesis are unaffected (Toper et al, 1981). They propose cell death may be due to excess 5'-dTMP decreasing the intracellular adenylate charge below the critical value for survival, and/or the intracellular accumulation of

deoxythymidine. High concentrations also induce mutations. However, strains deficient in RAD6, whose product is required for error-prone repair (Lawrence et al, 1970), do not show 5'-dTMP induced mutation (Barclay & Little, 1981). They believe fluxes of thymidine nucleotides may diminish the fidelity of DNA replication.

While thymidylate starvation or limitation does not induce mutation in nuclear genes of Saccharomyces cerevisiae, it greatly increases the frequency of mitotic recombination (Kunz et al, 1980).

When temperature sensitive tmp mutants are shifted from the permissive to restrictive temperature, nuclear dense bodies, (NDB) are seen. These are normally found during early meiotic development. Thymidylate stressed cells may activate parts of the meiotic pathway or conversely cells on sporulation medium may sense and respond to reduced thymidylate levels, together with other stimuli by entering the meiotic pathway (Moens, Barclay & Little, 1981).

The isolation and characterisation of the tmp mutants in yeast offered the opportunity to use the technique Pritchard and his co-workers developed on thy$^-$ bacterial mutants.

The aims and objectives of the work reported here were, (1) to develop a specific method for measuring S phase, using auto-radiography, (2) to elongate the S phase without affecting growth, and (3) to see what affect this had on other cell cycle and growth events.

RESULTS

Affect of 5'-dTMP on growth and morphology

Cells grown in medium N plus 5'-dTMP were deemed to be in a steady state if τ and B remained constant over a range of cell densities at 36°C. τ was measured as particle counts in an electronic particle counter, after routine sonication of the cell sample. At least 1000 cells were counted microscopically to determine cell morphology.

Figure 11 shows the growth curves for cells with 5'-dTMP in the media at concentrations of 50, 15 and 7.5 (μg/ml). Final cell number was similar for all three concentrations; 50 and 15 (μg/ml) had very similar doubling times while 7.5 was longer; the lag phase was longer the lower the 5'-dTMP concentration. The viability curve for 7.5 was parallel to the cell number curve (as was the case for all concentrations above this value), but below this concentration viability was reduced.

The inset shows cell number and viability for cells that had been grown in 50 μg/ml 5'-dTMP to mid-exponential phase, then filtered, washed and resuspended in medium N minus 5'-dTMP at 36°C. There was an approximately 66% increase in cell number, followed by an exponential decrease in cell viability. A similar result was obtained with cells treated identically taken from a stationary phase culture at 36°C, or from a mid-exponential or

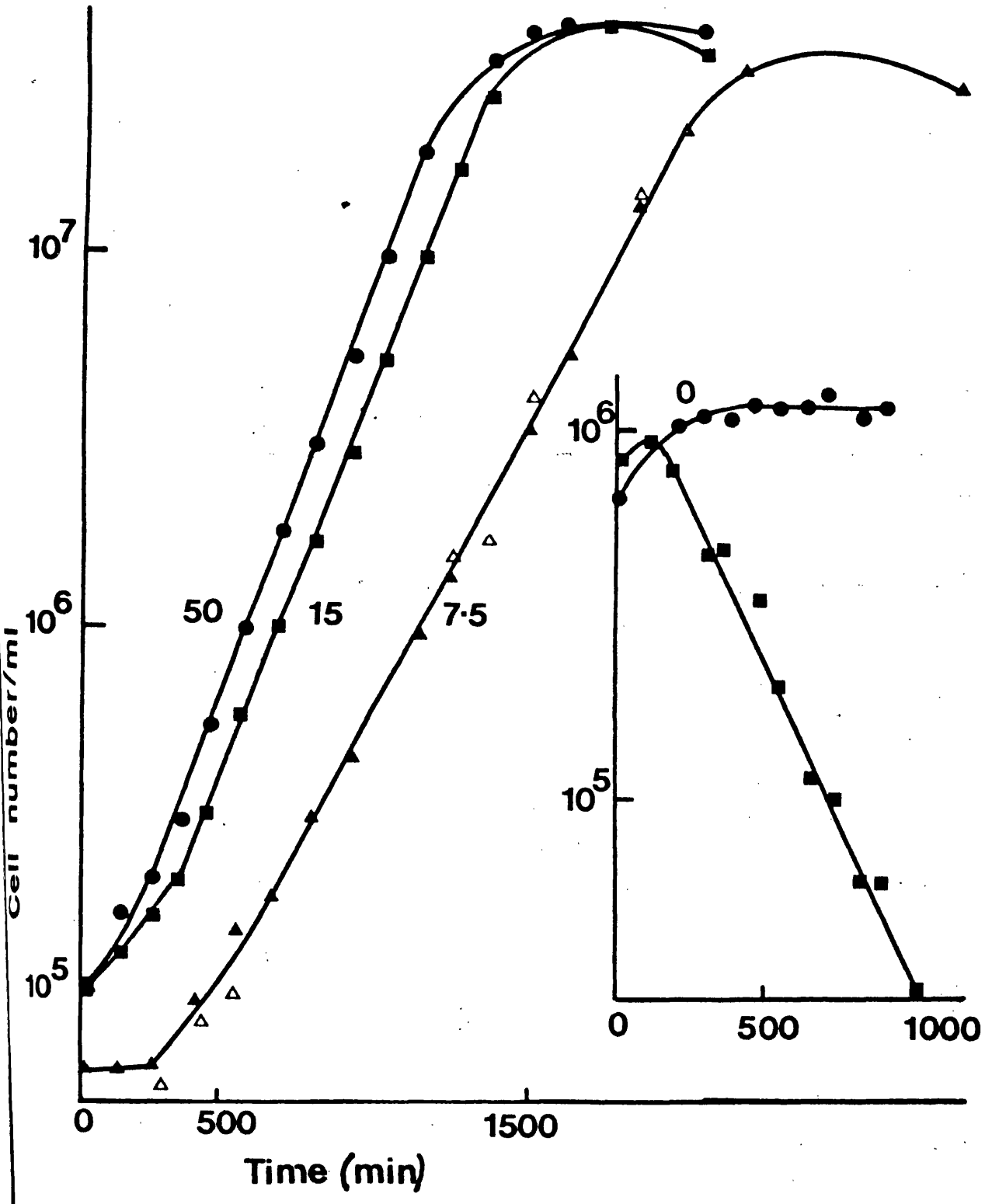


Fig11. Growth curves of ID in different 5′-dTMP concs (μg/ml). (△ viability). Inset. Cell number and viability in the absence of 5′-dTMP.

stationary phase culture at 23°C. 50% of cells arrested with the dumb-bell morphology characteristic of "Thymine-less death" (TLD), (Game, 1976), while others arrested with more abberrant morphologies showing much elongated buds (Figure 12).

Table 8 shows τ, final cell number, B and the percentages of cells showing TLD and abnormal or elongated morphologies. Over a concentration range of 250-15 µg/ml 5'-dTMP, τ remained constant at around 143 minutes, although there was some variation. At lower concentrations, τ increased. B was constant over the range 250-40 µg/ml 5'-dTMP but increased at lower concentrations. At 7.5 µg/ml there was a large increase in τ, yet B was similar in duration to 10 µg/ml.

Using the characteristic morphology of cells undergoing "TLD" as a measure of viability, microscopy revealed no significant loss at low 5'-dTMP concentrations. As the 5'-dTMP concentration decreased, the percentage of abnormal and elongated cells increased from less than 1% at 50 µg/ml to 18% at 7.5 µg/ml. Figure 12 shows some of the major types, cells with an elongated tubular bud, cells with their bud in the centre of a "bean-shaped" cell, and cells showing secondary bud formation.

The theoretical thymidylate concentration necessary for one round of DNA replication has been calculated to be 1.8×10^{-1} nmol per 10^7 cells by Toper et al (1981), or 0.00058 µg/ml. The concentrations used in the experiments vastly exceeded this value.

Figure 12. Abnormal morphologies at low 5'-dTMP concentrations.

(a) A cell with a elongated bud. (b) A cell with its bud in the centre of a "bean-shaped" cell. (c) Cells showing secondary bud formation.

a.

b.

c.

Table 8. Effect of 5'-dTMP concentration on cellular morphology.

5'-dTMP conc. (μg/ml)	Doubling time (min.)	Final cell number ($x10^7$)	Budded period (min.)	Morphology (%)		
				Thymine-less death	Abnormal cells	Elongated cells
250	142	NM +	101	0.5	0.2	0.1
100	138	5.9	96	0.3	0.0	0.7
100	145	NM	101	0.4	0.4	0.1
75	141	NM	99	0.9	0.0	0.6
50	144	4.5	98	0.5	0.2	0.0
50	136	NM	98	0.5	0.5	0.3
50	142	4.6	102	0.6	0.0	1.3
40	148	NM	104	NM	NM	NM
40	133	4.0	101	0.5	0.9	0.3
30	149	NM	115	0.8	2.8	2.5
25	140	5.0	109	1.9	1.0	1.0
20	144	4.2	114	0.4	1.4	7.2
20	146	5.0	115	1.4	4.0	5.0
15	140	3.7	115	0.6	2.1	9.2
15	145	NM	112	0.1	2.2	5.5
15	148	NM	120	0.6	3.7	9.8
15	145	4.5	113	0.9	2.7	3.8
10	169	NM	139	1.0	2.1	11.1
10	165	4.2	131	0.5	3.5	NM
7.5	214	3.8	135	0.4	7.7	10.4

+ Not Measured

It was vital that the 5'-dTMP concentration did not significantly alter during the growth of the culture. Four pieces of indirect evidence suggested that this was true. (1) Cell number increased exponentially, with cellular parameters including B remaining constant. (2) Final cell number was constant over the concentration range studied. (3) At 23°C final cell number was unaffected by the presence or absence of exogenous 5'-dTMP and was similar to that at 36°C. (4) Most importantly a doubling in the glucose concentration doubled the final cell number, and halving the glucose concentration halved the final cell number (Table 9) - glucose was limiting for growth and causing cells to enter stationary phase, not depletion of 5'-dTMP in the media.

Table 10 shows the mean volume at bud emergence of cells of different genealogical ages at different concentrations of 5'-dTMP. There was the characteristic increase in size at each genealogical age (Johnston et al, 1979), but also as the 5'-dTMP concentration decreased, the mean volume at bud emergence at corresponding genealogical ages increased dramatically.

Cell cycle parameters

The aim of the project was to increase S by limiting 5'-dTMP, a monomer for DNA synthesis, but without affecting τ.

The budding cycle and the DNA-division cycle of Saccharomyces cerevisiae are on separate pathways for most of the cell cycle but converge for cytokinesis and cell separation (Hartwell, 1974). If

Table 9. Final cell number as a function of glucose concentration.

5'-dTMP conc. (μg/ml)	Glucose conc. (g/l)	Final cell number + ($x10^7$)
50	20 (normal)	5.19
50	40	8.85
50	10	2.70

+ determined by particle counter

Table 10. Volume at bud emergence of cells of different genealogical ages at different 5'-dTMP concentrations.

5'-dTMP conc. (μg/ml)	Volume at bud emergence x (μm^3)				
	D+	P_1	P_2	P_3	Pn 3
50	27.1	33.5	39.4	42.2	64.6
25	35.1	40.5	47.3	48.5	66.5
20	40.0	47.9	50.8	56.5	65.1
15	40.2	49.7	51.3	65.5	70.9
7.5	65.2	69.8	83.4	89.3	105.4

+ Genealogical age was determined by bud scar analysis.

x Volume measured by a split image eye piece.

S is elongated, thus delaying division, B would be elongated. Because growth is by budding, division is normally asymmetrical, with the daughter being born smaller than the parent. The daughter cell thus needs a longer period of growth before reaching the critical size necessary for start (Johnston, Pringle & Hartwell, 1977). If B is elongated the daughter will remain attached for a longer period of time, acquire a larger volume, and thus be larger at birth and need less time before reaching the critical size and initiating the next division. This will manifest itself as a shorter unbudded period.

Estimates of D, P and B were calculated as described in Chapter 2. In Table 11 the lengths of, B, P, D, P-B, D-B and the ratio of D to P as a function of the 5'-dTMP concentration are shown.

B was constant over the concentration range 250-40 µg/ml but increased from 100-115 minutes as the concentration dropped from 40-15 µg/ml. τ remained constant over this range. At the lower concentrations the parent cycle time increased while the daughter cycle time decreased, hence the ratio of D:P decreased with decreasing concentration until at 7.5 µg/ml the value reached 1.

Figures 13, 14 and 15 show graphically the increase in B, the corresponding decrease in the unbudded period of parents and daughters and the cycle time of parents and daughters, as a function of the 5'-dTMP concentration.

Table 11. Duration of cell cycle parameters as a function of 5'-dTMP concentration.

5'-dTMP conc. (µg/ml)	Duration of phase (min.)						
	+τ	B	P	D	P-B	D-B	D:P
250	142	101	131	153	30	53	1.17
100	138	97	124	152	29	55	1.22
100	145	102	133	160	32	58	1.20
75	141	99	128	155	30	56	1.21
50	144	98	135	150	30	45	1.11
50	136	98	128	143	35	52	1.12
50	142	102	128	156	26	54	1.22
40	148	105	130	169	25	64	1.29
40	133	101	122	142	21	43	1.16
30	149	115	140	156	25	40	1.11
25	140	109	132	147	22	36	1.11
20	144	114	138	150	24	36	1.08
20	146	115	142	149	26	34	1.05
15	140	115	130	148	15	34	1.14
15	145	112	135	155	23	44	1.15
15	148	120	139	155	18	36	1.12
15	145	113	142	148	26	35	1.04
10	169	139	164	174	25	35	1.06
10	165	132	162	168	30	36	1.05
7.5	214	132	214	214	79	79	1.00

+ τ - population doubling time
B - length of budded phase
P - parent cycle time
D - daughter cycle time

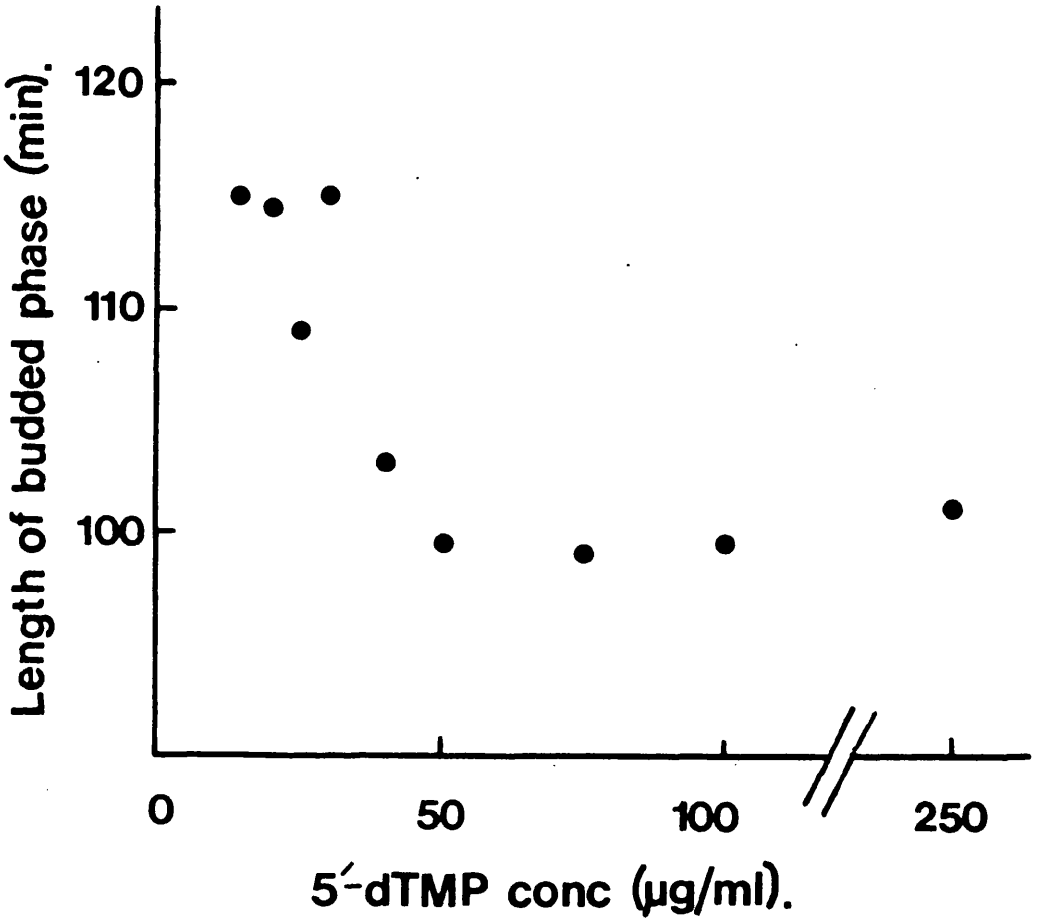


Fig13. Length of budded phase as a function of the 5´-dTMP conc.

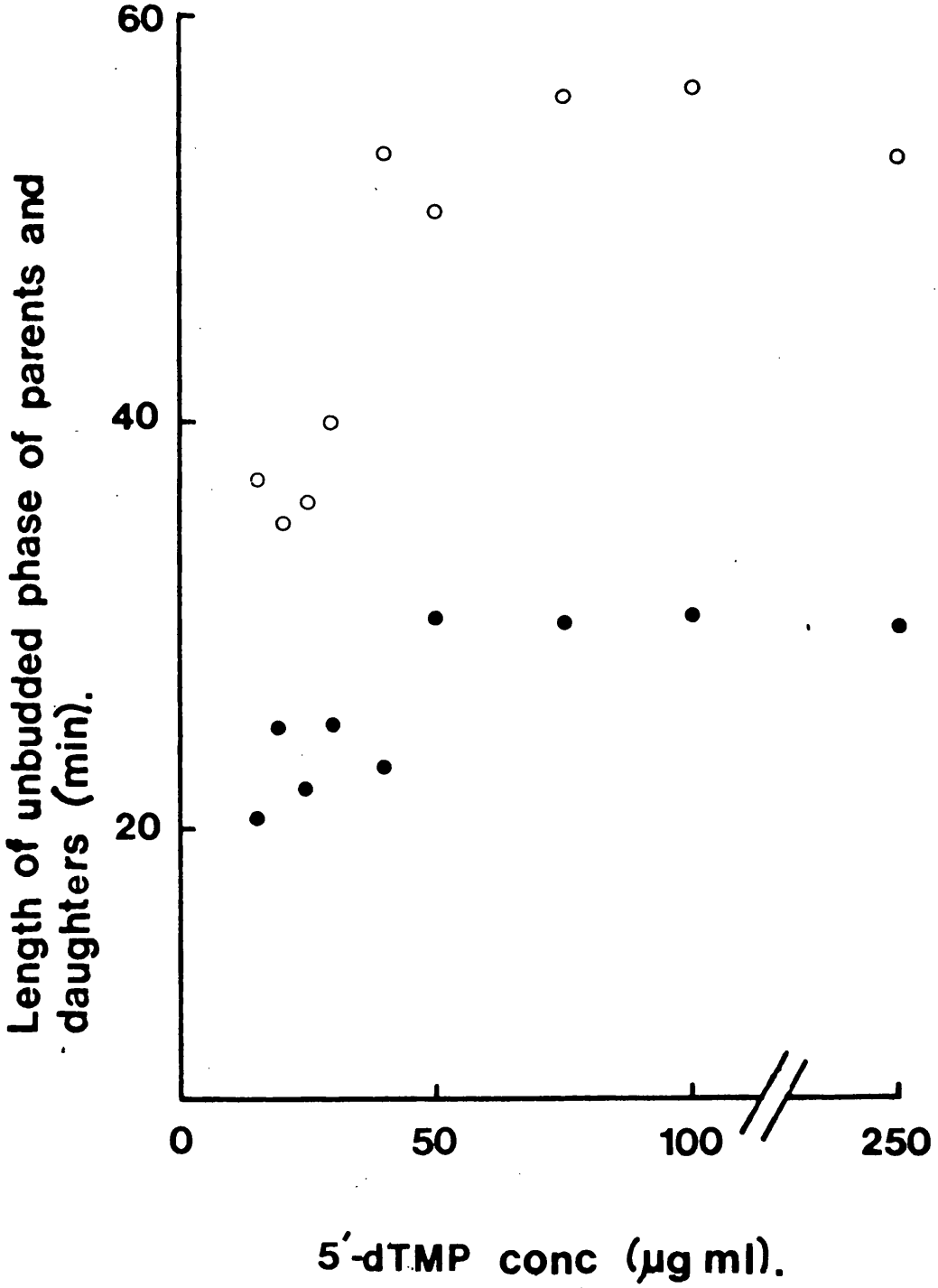


Fig 14. Length of unbudded phase of parents (•) and daughters (○) as a function of the 5′-dTMP conc.

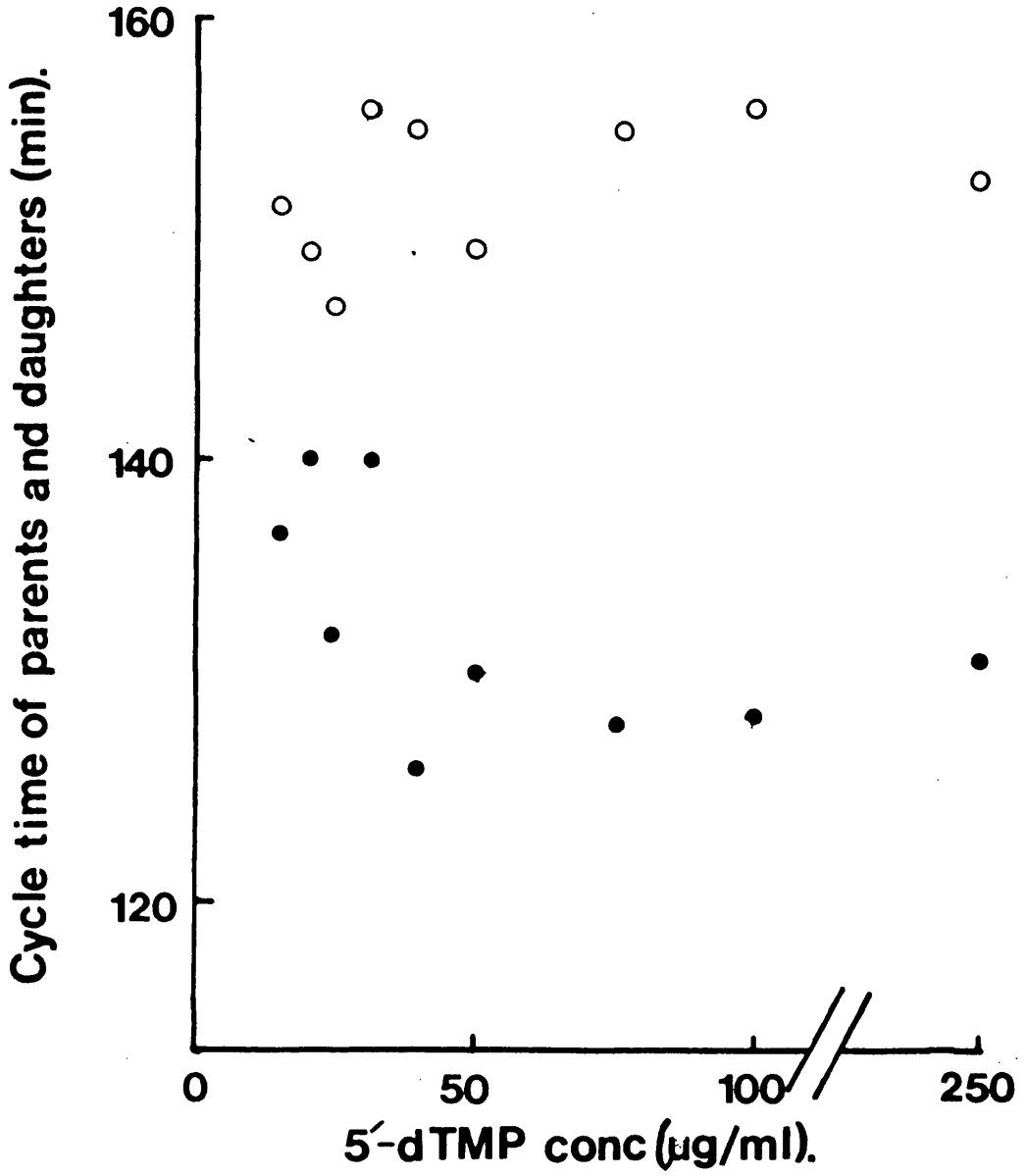


Fig15. Cycle time of parents(•) & daughters ○ as a function of the 5´-dTMP conc.

The increase in the budded period and decrease in the unbudded period gave strong indirect evidence for a lengthening in S phase caused by limitation of 5'-dTMP.

Estimation of cell cycle phases by nuclear morphology

The G_1 period starts after mitosis not with cytokinesis and bud emergence as a result of decreased 5'-dTMP concentration may have resulted from an expansion of the interval between mitosis and cytokinesis. This can be tested by examining nuclear morphology with the dye DAPI.

It was possible to determine four types of morphology - unbudded with a single nucleus, budded with a single nucleus, cells showing nuclear division (these are taken to be in mitosis), and binucleate cells. The latter morphology are cells after mitosis but before cell division and are in the G_1* phase of the cell cycle. Using the age distribution it was possible to calculate, from the frequencies of each class, the time spent in each stage as a function of the 5'-dTMP concentration (Table 12).

The results are plotted in Figure 16. G_1* and nuclear division were relatively constant over the whole concentration range. The increase in B at the lower concentrations was due to cells spending a longer time budded with a single nucleus. There was a decrease in the time spent unbudded with a single nucleus.

Initiation of S has been reported to be concomitant with, or

Table 12. Duration of cell cycle phases by DAPI analysis.

5'-dTMP conc. (μg/ml)	τ (min.)	Cell cycle time (min) spent			
		Unbudded with a single nucleus	Budded with a single nucleus	Nuclear division	Binucleate
500	144	48	67	10	19
100	138	47	59	9	23
75	141	46	70	7	18
50	142	45	67	9	21
25	143	36	78	10	19
20	144	28	81	11	24
15	140	30	79	11	20
10	169	28	106	11	24

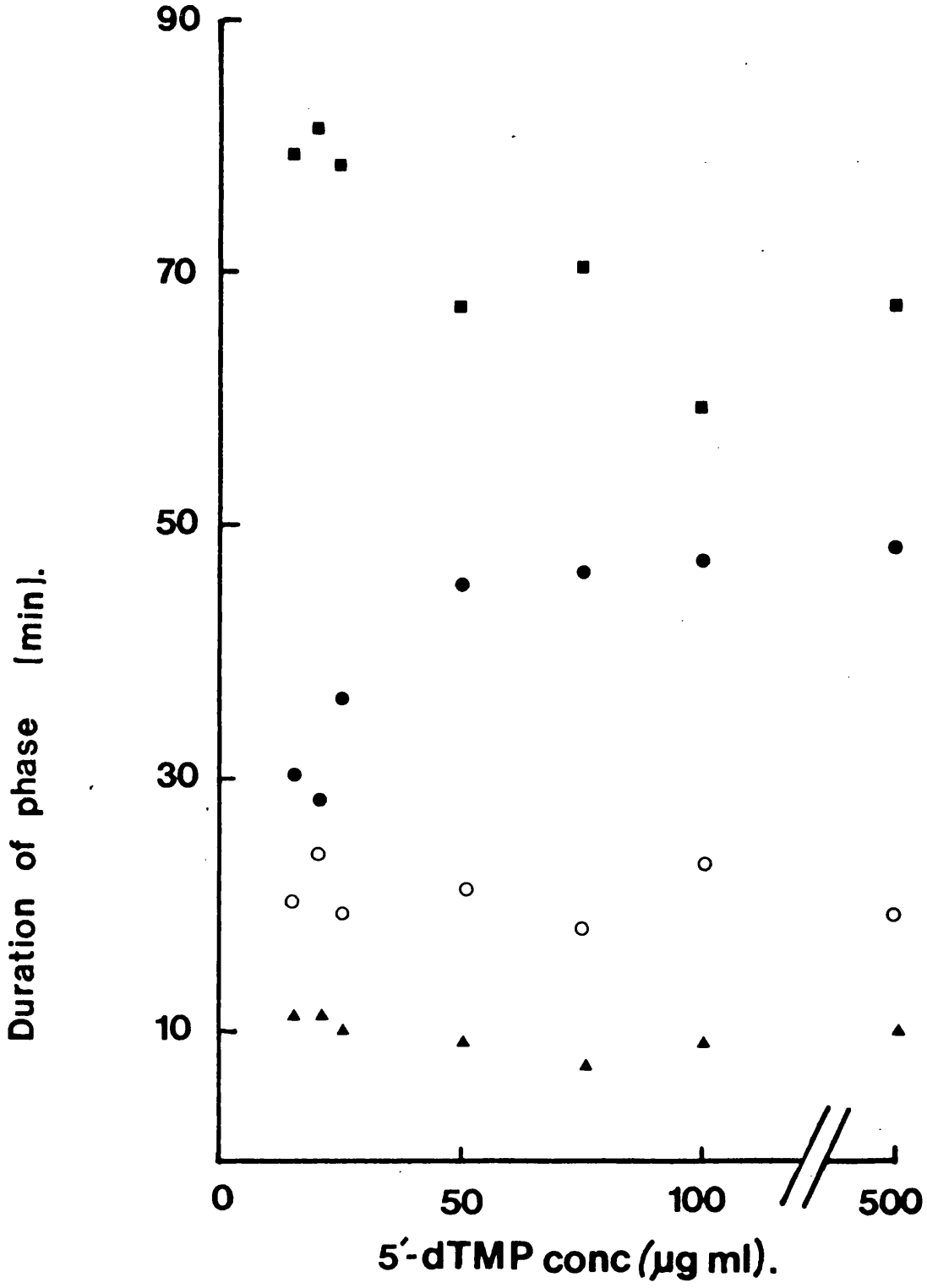


Fig16.Cell cycle phases as a function of the 5′-dTMP conc. Budded with a single nucleus(▪),unbudded with a single nucleus(•), nuclear division(▲) and binucleate cells(○) .

to occur before, bud emergence (Barford & Hall, 1976; Rivin & Fangman, 1980a; Slater, Sharrow & Gart, 1977). Thus an increase in the fraction of cells budded with a single nucleus supported the experimental hypothesis that S was being lengthened and G_1 shortened by the decreasing 5'-dTMP concentration. An increase in G_2 could not yet be ruled out. Nuclear division and cytokinesis occupy a constant time interval irrespective of the 5'-dTMP concentration. Although dependent upon completion of S (Hartwell, 1974), the length of nuclear division and cytokinesis seem to be independent of the length of S.

α-Factor execution point

Yeast cells are sensitive to mating pheremones at start (Hartwell, 1974), and thus the point of commitment in the yeast cell cycle can be detected. When exposed to α-factor, sensitive cells fail to bud and assume a characteristic morphology, a "shmoo" (Hartwell, 1974). Start can be determined by counting the number of unbudded cells that go on to form buds in the presence of mating factor (Rivin & Fangman, 1980a; Singer & Johnston, 1981).

Cells in mid exponential phase were rapidly collected, sonicated and overlaid onto medium N plus 5'-dTMP agar slides containing α-factor. Using time-lapse photomicroscopy the fraction of unbudded insensitive cells can be counted and used to calculate the α-execution point. The fraction of the cell cycle that is past the α-execution point is, $\frac{\log (1 + F_B + F_{IUB})}{\log 2} \tau$,

where F_B is the fraction of budded cells and F_{IUB} the fraction of insensitive unbudded cells.

Table 13 gives the α-execution point, the pre-start period of parents and daughters and the period from start to bud emergence.

At the lower concentrations of 5'-dTMP start occurred slightly earlier in the cell cycle and the period from start to bud emergence decreased. The pre-start period of parents increased while that of daughters decreased. The increase in P and decrease in D (Table 13) both appeared to be in the pre-start period.

A decrease in the interval from start to bud emergence, at lower concentrations, was expected if S was being lengthened at the expense of a shorter G_1.

Step-up experiments

If S was increased and G_1 decreased as a result of low 5'-dTMP concentrations limiting the DNA replication rate, an addition of a high concentration of the nucleotide under limiting conditions should increase the rate of DNA replication so reducing the length of S and the cell cycle time, until the cells adjust to their new environment and reach a new steady-state.

Cells were grown in a limiting concentration of 5'-dTMP (15 µg/ml). When the culture had reached steady-state growth,

Table 13. Start point as a function of 5'-dTMP concentration.

5'-dTMP conc. (μg/ml)	Cell cycle parameter (min)				
	τ	α-execution point +	Pre-start		Post-start to bud emergence
			P	D	
50	142	123	4	33	24
20	144	128	10	21	14
10	169	155	8	19	17

+ Time to division

The lengths of the pre-start period and post-start periods were determined by taking the values of D, P and B from Table 10. 100-200 cells were counted.

half of the cells were removed and 5'-dTMP was added in excess to reach a final concentration of 500 μg/ml.

In Figure 17, increase in cell number is plotted against time for both the control and the step-up experiment. The results from two experiments are shown. For the first 40 minutes increase in cell number of the step-up culture was parallel to that of the control. This was followed by a more rapid increase up to 100 minutes, and then a 30 minute lag after which cell number increased again in parallel to the control.

In Figure 18, the percentage of budded cells is plotted against time for the two cultures. The percentages were similar for the first 40 minutes at 80% followed by a rapid decrease in the step-up culture to 55% at 70 minutes. The percentage then rose to its new steady-state value of 65% after 100 minutes.

The results agree with the experimental rationale. In the step-up culture increase in cell number was initially parallel to the control because cells that were post-S (G_2, M and G_1*) should be unaffected by the higher concentration and divide at the old rate (rate maintenance). The value of forty minutes should be equal to G_2, M and G_1* and is in good agreement with the estimates from nuclear staining which gave the length of M and G_1* alone at 31 minutes (Table 12). The rapid increase after this time occurred because cells were being accelerated through S in the step-up culture, and S was no longer the rate limiting step in the cell

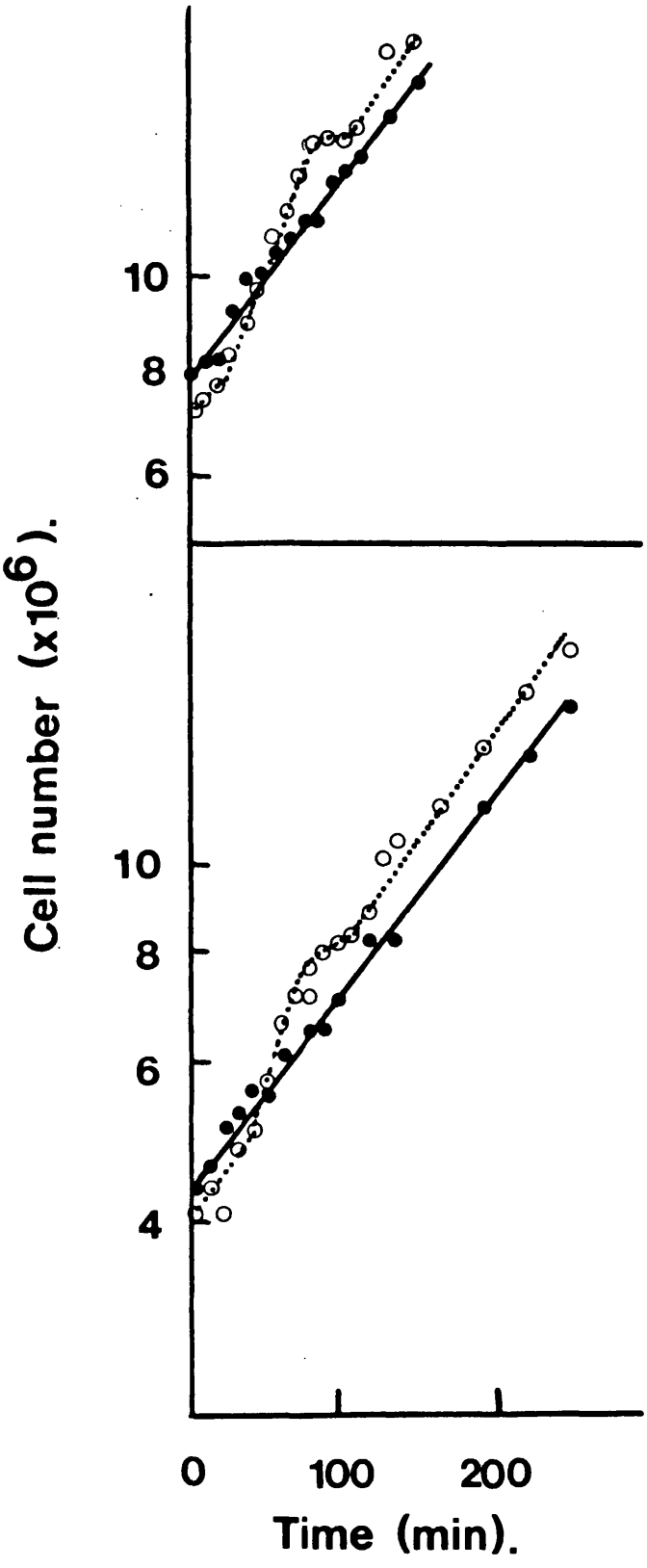


Fig17. Increase in cell number after a step-up from 15 to 500 µg/ml 5′-dTMP (○). Control (●). Two experiments are shown.

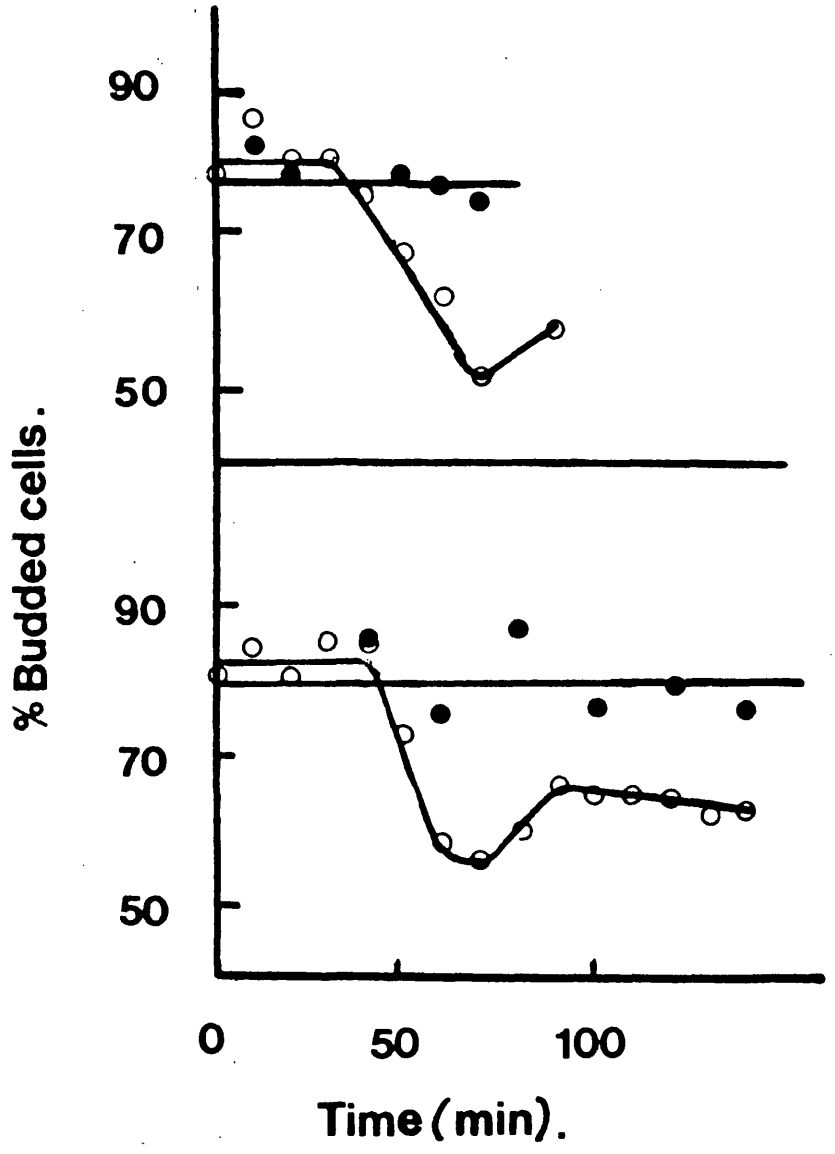


Fig 18. Percentage of budded cells after a step-up from 15 to 500 µg/ml 5′-dTMP(•). Control(o). Two experiments are shown.

cycle.

The newly divided cells caused by the addition of excess 5'-dTMP will be smaller because S, G_2, M & G_1* were reduced in length. These need to grow before they can reach the critical size necessary for traverse of start (Johnston, Pringle & Hartwell, 1977). This will result in a transient lag in cells entering the next DNA-division cycle and manifest itself as a lag in cell number increase. The lag approximates the G_1 period. Parents are born greater than the critical size necessary for start and can re-enter the DNA-division cycle almost immediately after cell separation (Lord & Wheals, 1981). Parents and daughters will respond differently to the reduced cycle time and this would explain the lack of synchrony in the lag, particularly in the lower figure.

The accelerated rate of cell division after addition of excess 5'-dTMP should equal the duration of the S phase and the value obtained of 50 is in good agreement with the autoradiography data (see below).

The rapid increase in the rate of division was reflected by the rapid decrease in the percentage of budded cells (Figure 18). The culture then reached a new steady-state value of 65%, normal for the strain under non-limiting 5'-dTMP concentrations. The overshoot is due to the partial degree of synchrony.

The step-up experiment has provided further indirect evidence that 5'-dTMP limitation was increasing the length of S at the expense of G_1.

5'-dTMP limitation in different media

At low 5'-dTMP concentrations some cells growing in medium N plus glucose showed abnormal morphologies. Presumably at these concentrations cells cannot take up sufficient 5'-dTMP to support a normal length DNA replication period, and this caused a lengthening of S which affected the control and timing of normal growth events.

This and other experiments showed that by limiting the 5'-dTMP it is possible to alter the timing of the cell cycle events without altering the population doubling time. Another widely used method for changing the timing of cell cycle events is to alter the population doubling time by using different growth media. The 5'-dTMP mutants offer the chance to use both techniques together.

If the cause of the abnormalities at the low concentrations is that the cycle time is not allowing sufficient and correctly sequenced growth, then if τ is increased while 5'-dTMP concentration remains constant, the increase may allow sufficient growth and the frequency of abnormal cells should decrease. Conversely decreasing the cell cycle time should magnify the effect of 5'-dTMP limitation and lead to even more abnormal cells being produced.

Medium N with mannose as the carbon source was used to slow down the growth rate, and YEPD was used to achieve a faster growth rate.

Mannose slowed the growth rate down to 245 minutes. In Table 14, B, P, D, D:P and the percentage of abnormal cells are shown as a function of the 5'-dTMP concentration.

At 50 µg/ml growth was normal. At 20 µg/ml there was no significant increase in B, in contrast to medium N plus glucose (Table 8), 4% of cells were abnormal, half the value in medium N plus glucose. At 10 µg/ml there was an increase in both B and the percentages of abnormal cells. The limiting concentration of 5'-dTMP, as measured by an increase in B and the percentage of abnormal cells, was lower at the slower growth rate.

YEPD medium supported fast growth rates with other strains (Lord & Wheals, 1980). At the permissive temperature the population doubling time was 140 minutes. YEPD plus 50 µg/ml 5'-dTMP at the restrictive temperature had a doubling time of 210 minutes, with over 80% of the cells budded and 20% with abnormal morphologies. Increasing the 5'-dTMP concentration to 500 µg/ml gave a population doubling time of 140 minutes, with 60% budded cells and less than 1% with abnormal morphologies. At the faster growth rates higher 5'-dTMP concentrations are needed to support normal growth.

There are two possible explanations for the results in YEPD.

Table 14. Cell cycle parameters in mannose medium.

5'-dTMP conc. (µg/ml)	Parameter (min)				D:P	Abnormal cells (%)
	τ	B	P	D		
50	245	111	157	345	2.2	1
20	249	115	167	314	1.9	4
10	259	180	223	287	1.3	6.5

Firstly, cells cannot take up sufficient 5'-dTMP for normal growth, and secondly, the more complex media might have affected the 5'-dTMP perhaps by enzymatic degredation or binding. Little & Haynes (1979) found that lower 5'-dTMP concentrations are needed for optimal growth in minimal media compared to YEPD. In contrast to their report inhibition of growth by high 5'-dTMP concentrations was not apparent even at a concentration of 500 μg/ml (Table 12).

Measurement of S phase

The length of S phase at different 5'-dTMP concentrations was examined by preparing whole cell autoradiographs from cell cultures pulsed with labelled 5'-dTMP.

Label uptake

For the mutants to be used in labelling experiments it was necessary to show that labelled 5'-dTMP was incorporated during exponential growth.

Figure 19 shows the kinetics of label uptake. 25 μCi/ml of ^{3}H5'-dTMP was added to a mid-exponential phase culture of strain 1D in medium N plus 50 μg/ml of unlabelled 5'-dTMP. The culture was sampled at intervals, and the number of base stable, acid precipitable counts measured. The uptake curve can be extrapolated to show linear incorporation after 8 minutes, giving a labelling period of 7 minutes. The curve is similar to those for adenine and uracil incorporation (Johnston & Williamson, 1978; Rivin & Fangman, 1980a).

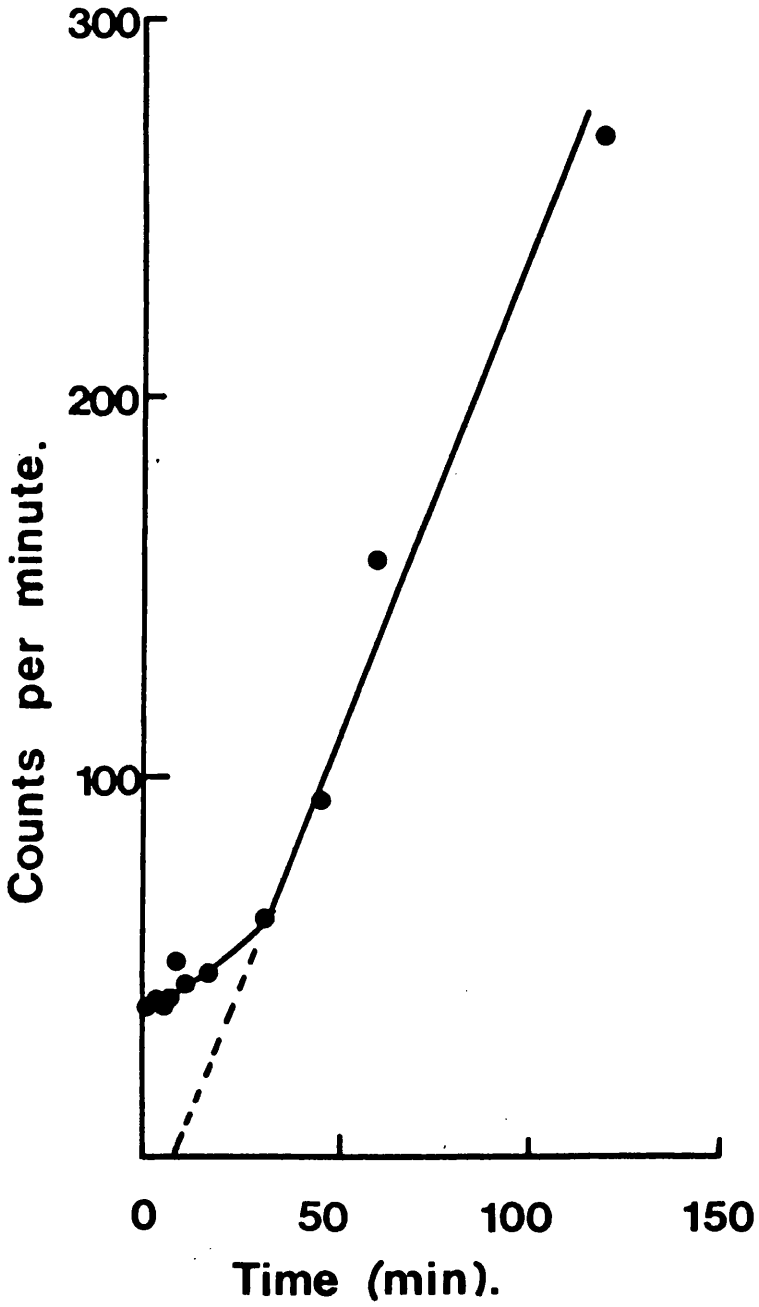


Fig19. Kinetics of label uptake.

Pulse labelling experiments

An exponentially growing culture was exposed briefly (15 minutes) to radioactive 5'-dTMP (^{3}H 5'-dTMP at 25 μCi/ml), fixed and taken for autoradiography (see Chapter 2). When sufficiently exposed (2-6 weeks) the samples were scored for the uptake of label and the presence or absence of buds. Cells in S phase at the time of the pulse will incorporate the radioactive thymidine into their DNA.

To calculate the percentage of labelled cells it is necessary to consider the effect of grain count on both labelled and unlabelled cells (the background count). For these purposes the assumption is made that the counts will show a Poisson distribution

$$P(x) = \frac{e^{-m} m^{x}}{x!},$$

where x is the number of grains associated with a given cell and m is the average number of grains per cell.

With a large m value, almost all labelled cells will have more grain than background and the error in counting the percentages of labelled cells will be negligible. However, too high a value of m may result in cells having so heavy a grain density that the morphology is obscured. If m is low this problem will not arise, but a significant number of cells will show no more than the background number of grains. This is overcome by scoring all cells for the number of grains and plotting the frequency of different grain counts P(x) against the log Px(x!). The Poisson

distribution plots as a straight line and higher grain counts are used to determine the slope of the line and an extrapolation made to obtain the actual percentage of labelled cells with zero or only a few grains (Figure 20).

Table 15 shows the percentage of labelled budded or unbudded, and unlabelled budded or unbudded cells corrected by the Poisson analysis. m is also shown. Cells that were post S at the time of the pulse should not be labelled, and as bud emergence occurs before completion of DNA synthesis (Rivin & Fangman, 1980a; Barford & Hall, 1976) the duration of the cell cycle that is post S is given by the equation

$$PS = \frac{\ln (F_{BUL} + 1).\ \tau}{\ln 2} \qquad (a)$$

where PS = the duration of cycle post S i.e. G_2+M+G_1*

F_{BUL} = the fraction of budded unlabelled cells

The cells in S will be labelled by the pulse, and the time in S plus G_2, M and G_1* is given by

$$S + PS = \frac{\ln (F_{BUL} + F_L + 1).\ \tau}{(\ln 2)} \qquad (b)$$

where, S + PS = the duration of cycle in and post S

F_L = the fraction of labelled cells

Taking (b) from (a) will give the time spent in S phase, and taking (b) from τ the time spent in G_1.

Table 16 summarises the data from the pulse labelling experiments of cultures grown in 5'-dTMP concentrations of 50,15 and 10 µg/ml.

Figure 20. Poisson distribution of the number of grains per cell.

1000 cells were counted for each experiment, and scored for the number of associated silver grains (x). The data are plotted so that a Poisson distribution appears as a straight line. Higher grain counts are used to find the slope of the line, and an extrapolation made to obtain the true fraction of labelled cells among those cells with low grain numbers.

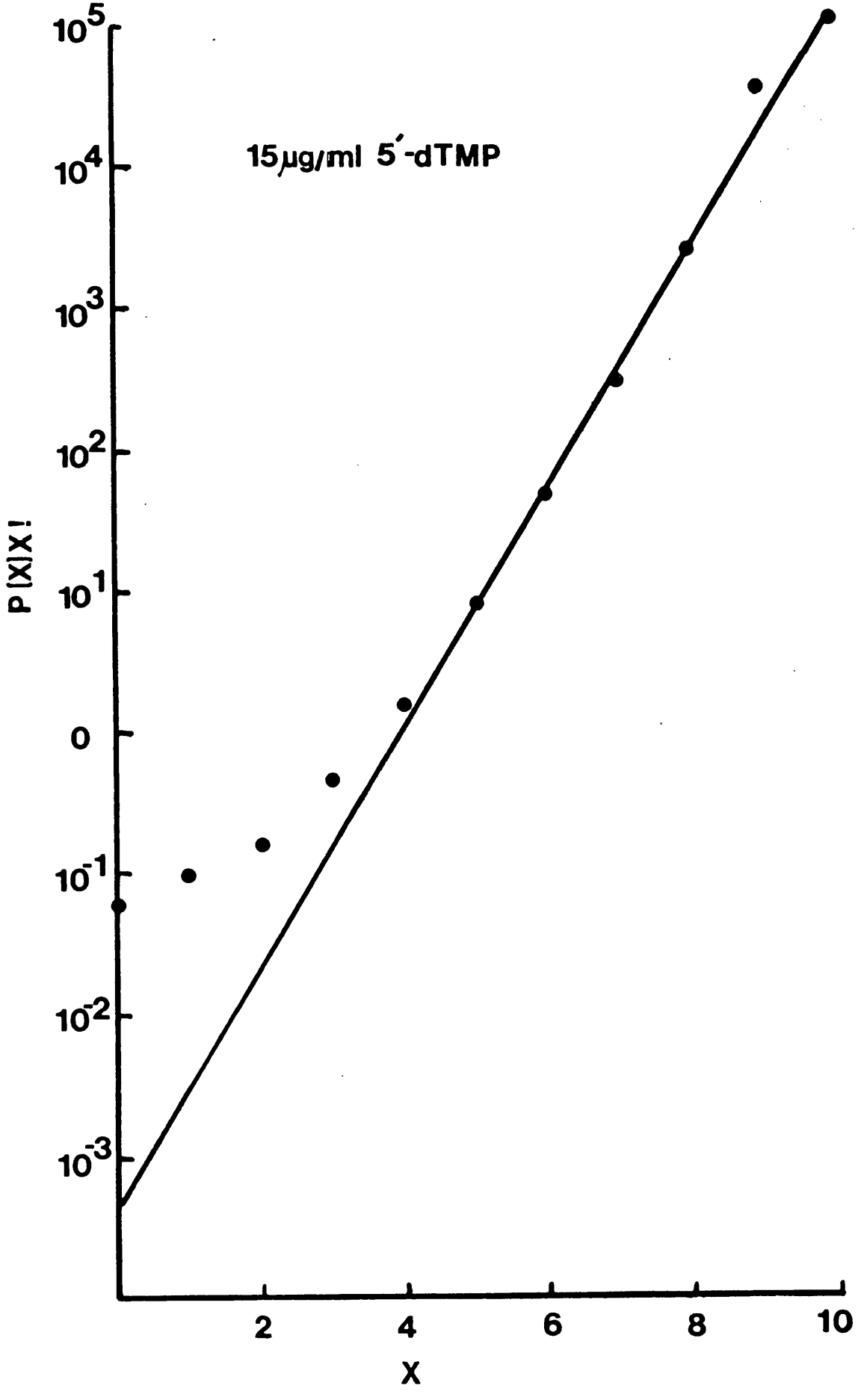

15µg/ml 5´-dTMP
P[X]X!
10^5
10^4
10^3
10^2
10^1
0
10^{-1}
10^{-2}
10^{-3}
2
4
6
8
10
X

Table 15. Percentage of cell types as a function of the 5'-dTMP concentration.

5'-dTMP conc. (µg/ml)	Cell type (%)					M
	Unlabelled unbudded	Labelled unbudded	labelled budded	Unlabelled budded	Total labelled	
50	33	10	22	35	32	2.10
50	26	10	21	43	31	2.12
50 +	21	8	23	40	31	2.06
15	9	20	51	20	71	7.19
10	8	30	36	26	66	4.92

+ Treated with RNase

M Mean number of grains per cell in a Poisson distribution.

Table 16. Duration of cell cycle phases measured by autoradiography.

5'-dTMP conc. (µg/ml)	τ (min)	Duration of stages (min) G_1	S	G_2+M+G_1*
50	140	36	43	61
50	142	28	40	74
50+	142	33	41	68
15	145	10	97	38
10	169	25	97	47

\+ Treated with RNase

To ensure that the label was incorporated specifically into DNA, autoradiographs were prepared from N plus 50 μg/ml 5'-dTMP media and some treated with RNase and some with DNase. From the table it is clear that RNase treatment had no noticeable affect on the percentage of labelled cells. The cells treated with DNase showed very little label and no heavily labelled cells were found. The label is specific for DNA as was shown by Brendel and Fäth (1974). The method gives reproduceable results since the three analyses at a concentration of 50 μg/ml 5'-dTMP gave constant values for S. For the autoradiography at 15 μg/ml 5'-dTMP, the pulse time was 20 minutes not 15. The result of this would be that more cells which have left S and entered G_2 during the pulse will be labelled, and the value of S lengthened by up to 5 minutes.

At the lower concentrations of 5'-dTMP there was clearly an increase in S, the value being more than doubled from 40 to 97 minutes. There was a decrease in G_1 as predicted, and also in G_2. The experiment gave proof that limiting 5'-dTMP causes a decrease in the rate of DNA replication.

In Table 17 the length of cell cycle phases at different 5'-dTMP concentrations is presented (the results are illustrated in Figure 22). The data from all the experiments were pooled. The measurement of start used for 15 μg/ml was one obtained for a concentration of 20 μg/ml.

The general trends with decreasing 5'-dTMP concentration are,

Table 17. Lengths of cell cycle phases (min.) as a function of the 5'-dTMP concentration (μg/ml)

5'-dTMP conc. (μg/ml)	Duration of cell cycle parameters (min.)											
						G_1						
	τ	P^+	D^+	$Start^+$	B^+	P	D	S	G_2	M	G_1*	iDS→BE
50	140	131	146	-	92	27	42	43	(------61-------)			12
50	142	127	156	124	97	13	42	40	44	9	21	17
50[x]	142	127	156	124	99	18	47	41	38	9	21	10
15	145	134	154	128"	122	-1	19	97	14	9	15	13
10	169	163	174	156	135	9	20	97	22	11	24	19

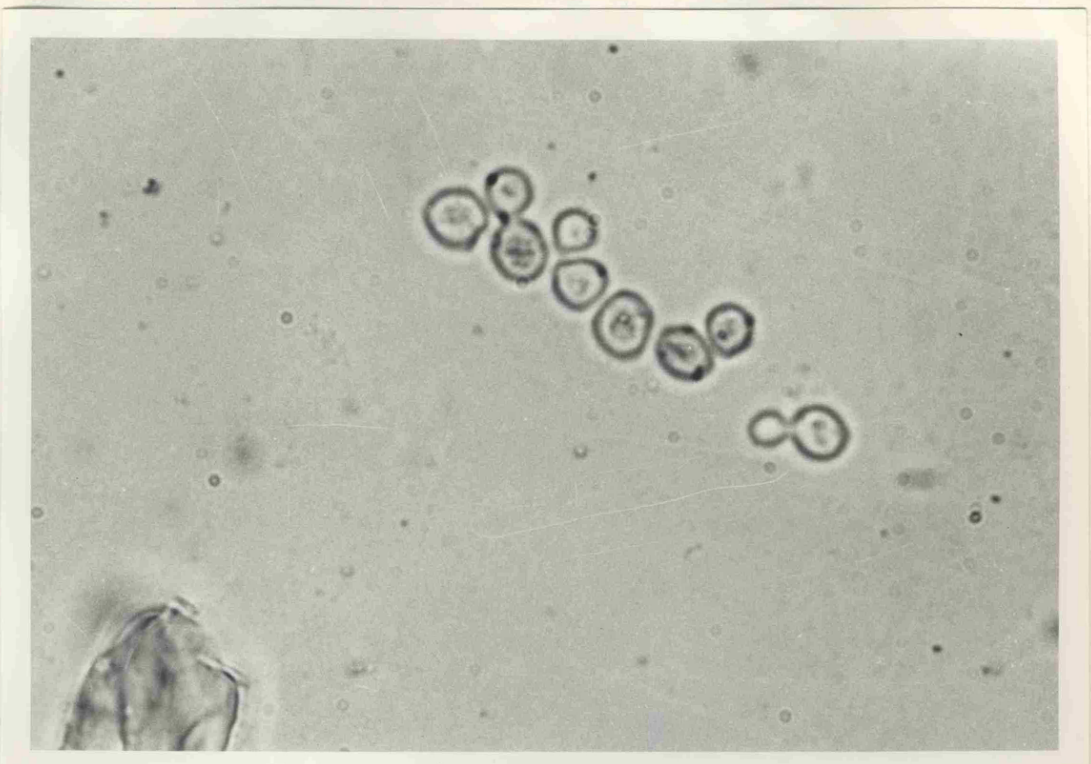
+ Point measured to division

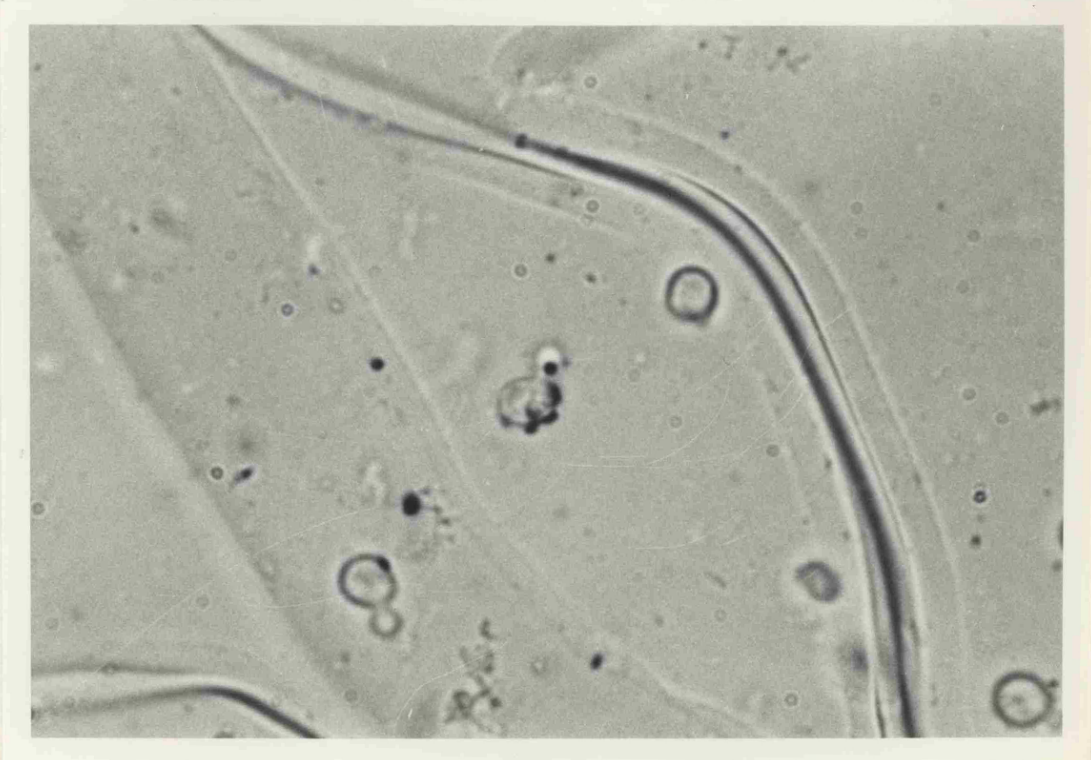
x RNase treatment

" Data taken from 20 μg/ml experiment

Figure 21. Saccharomyces cerevisiae cells labelled with ^{3}H 5'-dTMP

Cells were grown in medium N plus 50 µg/ml 5'-dTMP, and radiolabelled 5'-dTMP added to a concentration of 25 µCi/ml. (a) The picture illustrates all four classes of cell scored, unbudded unlabelled, unbudded labelled, budded unlabelled, and budded labelled. (b) A more densely labelled budded cell.





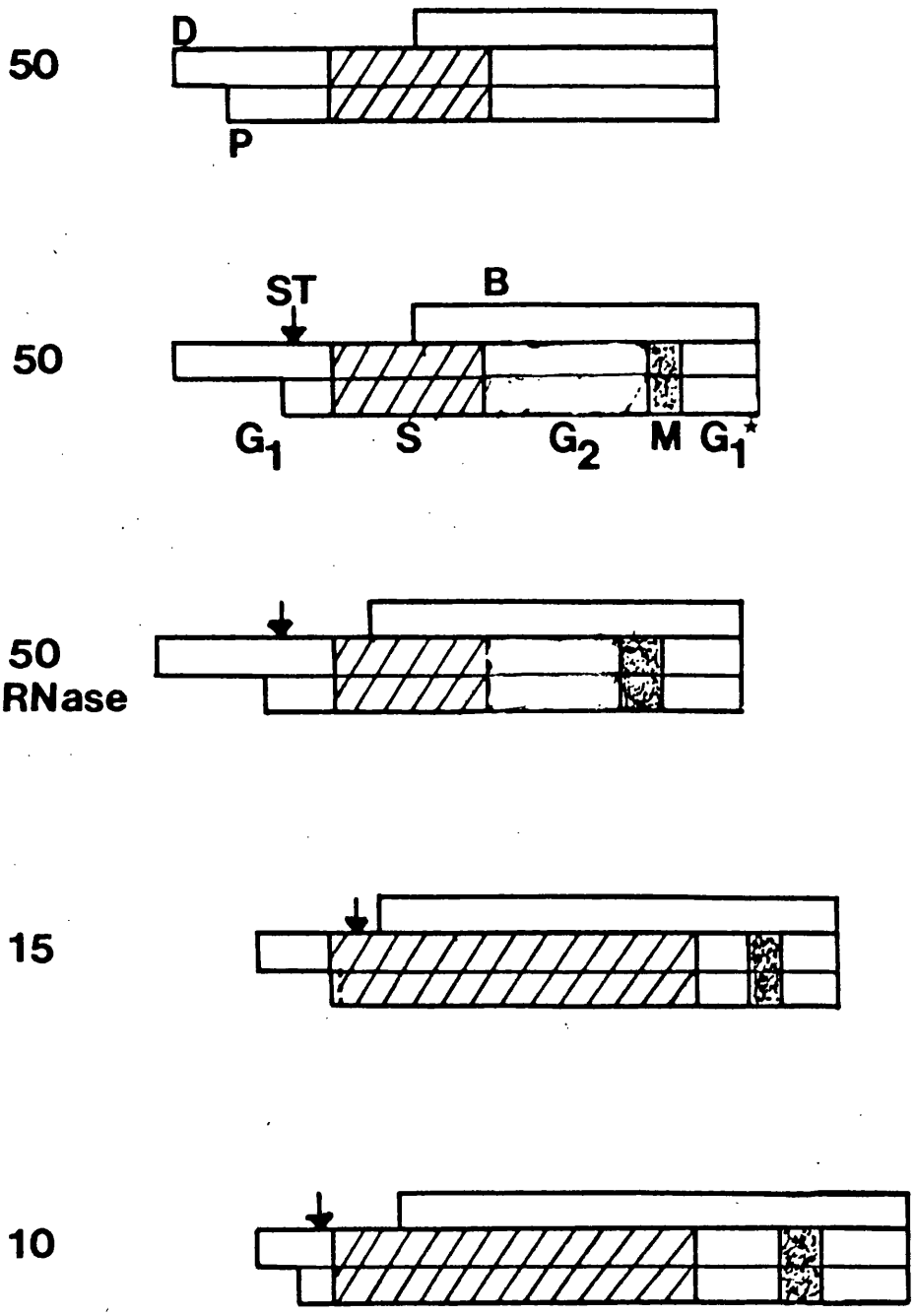


Fig22.Cell cycle phases as a function of the 5'-dTMP conc (µg/ml).

an increase in B and S, a decrease in G_1 of both parents and daughters, and a decrease in G_2. There was constancy of M and G_1*.

DISCUSSION

Evidence for an Increase in S

The major aim of the work was to elongate S, without altering τ, and to see what effect this had on the yeast cell cycle. It was hoped to accomplish this by reducing the amount of 5'-dTMP, an essential precursor for chain elongation, in the growth medium.

There were five pieces of indirect evidence to support an elongation of S, with an unaltered τ, at reduced 5'-dTMP concentrations; (1) The increase in the length of the budded phase, (2) the nuclear staining experiments, (3) the step-up experiments, (4) the increase in the percentage of cells with abnormal morphologies, and (5) the experiments where the glucose concentration was altered. Direct evidence was supplied by the autoradiography experiments.

Table 8 shows that decreasing the 5'-dTMP concentration from 250-15 μg/ml had no effect on the population doubling time. Over the range 50-15 μg/ml there was an increase in the length of the budded phase. The budding cycle and the DNA-division cycle of Saccharomyces cerevisiae are on separate pathways but merge for cytokinesis and cell separation (Hartwell, 1974) and an elongation in one would elongate the other. If S was elongated by 5'-dTMP limitation it may be illustrated by an increase in B.

Nuclear staining with the dye DAPI showed that as the 5'-dTMP

concentration was reduced the fraction of the cell cycle spent budded with a single nucleus, a morphology characteristic of cells in S and G_2, increased.

In the step-up experiment, a high concentration of 5'-dTMP was added to cells growing at a limiting concentration. Upon addition, after an initial lag there was an increase in the rate of cell division, which was predicted if the rate of DNA replication was suddenly accelerated.

The increase in the percentage of abnormal and elongated cells suggested that at reduced 5'-dTMP concentrations the normal course of growth events is disrupted, presumably caused by an increase in S.

5'-dTMP concentration must not significantly alter during the growth of the culture, or this may be a possible explanation for some of the experimental observations. A doubling of the glucose concentration, doubled the final cell number,a halving of it, halved the final cell number, showing that it was depletion of glucose not 5'-dTMP that caused cells to enter stationary phase, and that there was a constant supply of 5'-dTMP available for DNA synthesis during growth, far in excess of that required for a round of DNA synthesis.

The autoradiography method developed during this research allowed direct measurements of S to be made. At the non-limiting

concentrations of 50 μg/ml 5'-dTMP, three estimates of S gave a time for DNA replication of approximately 40 minutes (Table 16). Decreasing the 5'-dTMP concentration to 15 μg/ml increased the length of S from 40 to 97 minutes (with an unchanged τ). This value is a slight overestimate because the labelling time was 20 minutes instead of the usual 15 minutes. At a concentration of 10 μg/ml where τ was increased to 165 minutes, S occupied 97 minutes. At non-limiting concentrations S phase occupied 30% of the cell cycle, while at limiting concentrations this could be increased until it occupied over 60% of the cell cycle.

Rivin & Fangman (1980b) showed that under conditions of nitrogen limitation the increase observed in S phase was due solely to a reduction in the rate of fork elongation - at the slowest doubling times it was reduced four fold compared to the control. It is probable that the increase in S caused by limitation of thymidine, a monomer directly required in fork elongation, was also brought about by a reduction in the rate of elongation of the replicating strand.

Autoradiography

Another major aim of the research was to develop a method of measuring S phase using the 5'-dTMP mutants.

Label incorporation in strain 1D showed a similar uptake curve to other deoxyribonucleotides (Fig. 19) (Johnston & Williamson, 1978; Rivin & Fangman, 1980a). The labelling,

preparation for autoradiography and developing used established and reliable methods (see Chapter 2). By altering the amount of labelled thymidine offered it was possible to reduce exposure time for rapid results. A major advantage the mutants have over previous autoradiographic methods of measuring S is that labelled thymidine is specific for DNA, and no RNase treatment is necessary.

Three different analyses of the length of cell cycle phases in cells growing in medium N plus 50 μg/ml 5'-dTMP are shown in Table 16. The culture with a doubling time of 140 minutes was split and samples taken for RNase and DNase treatment. All three estimates of S were constant at approximately 40 minutes, or 30% of the cell cycle. The values for G_1 and G_2M & G_1* measured together, were also reasonably constant.

Two pieces of evidence showed that RNase treatment was unnecessary. Firstly, the same value of S was obtained regardless of whether RNase hydrolysis was applied. Secondly, after DNase treatment cells had very few grains, and counts above the background number of the control were rare.

S occupying 30% of the cell cycle falls within the experimental range previously obtained for cultures grown under non-limiting conditions, but the problems encountered in measuring S, e.g. ploidy, experimental conditions, strain differences and method of analysis, preclude a specific comparison.

Effect of Elongation of S on other Cell Cycle Parameters

Altering the timing of cell cycle events by varying the population doubling time has been well documented in Saccharomyces cerevisiae (Pringle & Hartwell, 1981). G_1 is the most flexible phase, and when τ is increased most of the increase occurs in this phase. Increase in S when τ is lengthened has only been reported under conditions of nitrogen limitation (Rivin & Fangman, 1980a; Johnston et al, 1980). G_2, M and G_1* are usually measured together, and Barford and Hall (1976) reported a three fold increase when τ was increased from 92 to 435 minutes. Slater et al (1976) reported a two fold increase with a three fold increase in τ. Johnston et al (1980) noted a similar trend.

In this project the timing of S phase has been altered, while τ was unchanged.

If τ was unaltered, and the length of S more than doubled, there must be a decrease in other cell cycle parameters. The results are summarised in Table 17. Nuclear staining with DAPI showed both M and G_1* to be constant over the experimental range. The decrease in the unbudded phase at lower concentrations indicated a reduction in G_1 (see results), and direct measurement at 15 µg/ml gave a greatly reduced G_1 of 10 minutes compared to 30 minutes at 50 µg/ml. At 10 µg/ml, G_1 lasted 25 minutes, still less than at 50 µg/ml but τ was increased to 169 minutes. The increase in τ at this concentration was due mostly to an increase in G_1. G_2 is more than halved in length at 15 µg/ml from 40 to 14

minutes, although this value is likely to be a slight under-estimate due to the increased labelling time. At 10 μg/ml G_2 was also significantly reduced.

Support for the autoradiography results was supplied by the step-up experiment which gave an estimate of G_2, M and G_1* at 15 μg/ml of approximately 40 minutes and a value for S under non-limiting conditions of approximately 50 minutes.

At the lower 5'-dTMP concentrations P was increased slightly and D decreased in comparison to the control, i.e. the ratio of P:D decreases. If the G_1 period is for growth only, an increase in B of 10 minutes should lead to a decrease in the unbudded period of parents and daughters of 10 minutes and P:D would remain constant. Parents are however born greater than the critical size necessary for start, and DNA-division cycle events commence within a few minutes of cell division (Lord & Wheals, 1981). α-factor experiments gave a pre-start period of only 4 minutes in parents grown at 50 μg/ml 5'-dTMP, that of daughters was 33 minutes. One explanation for the longer P would be that when S increased, the other cell cycle phases could only be decreased to a certain extent, after which start to cell division of parents must increase. However the α-factor experiments revealed that from 50-20 μg/ml the period from start to division increased only slightly, while the pre-start period of parents increased significantly. Post-start G_1 and the G_1 period as a whole are reduced in parents when 5'-dTMP becomes limiting for DNA synthesis. Both the pre-start and

post-start G_1 periods of daughters are reduced.

The increase in pre-start G_1 of parents may explain why at 10 μg/ml 5'-dTMP, τ was increased to 169 minutes and G_1 occupied 25 minutes. At 7.5 μg/ml 5'-dTMP τ increased to 215 minutes, division was symmetrical, yet the length of B remained the same as at 10 μg/ml. The increase in the unbudded period was indicative of an increase in G_1, in both parents and daughters, presumably in the pre-start period.

This proposal was not supported by the presence of cells showing secondary bud formation. In these cells start must have occurred well before cell separation for buds to appear prior to cell division. Perhaps a small percentage of cells in the population overcame the demands of thymidine limitation without the need for an increased pre-start period of parents. The method of measuring start should have been accurate as it has been widely used (Singer & Johnston, 1981; Rivin & Fangman, 1980a).

The most obvious candidate for causing the increase in pre-start G_1 is 5'-dTMP itself. The fact that, (1) exponentially growing cells placed in medium N minus 5'-dTMP arrested as doublets, characteristic of cells in S or later in the cell cycle, and not unbudded, characteristic of cells arrested at start, and (2) that stationary phase cells when placed in fresh medium minus 5'-dTMP initiated the DNA-division cycle and arrested as doublets, shows that 5'-dTMP is not measured at start.

A similar type of analysis on Saccharomyces cerevisiae was carried out by Singer and Johnston (1981). They used two methods to increase S, (1) drugs which inhibited DNA synthesis were added at reduced concentrations, and (2) a temperature sensitive mutant, cdc8, was grown at intermediate temperatures. They also reasoned that an increase in B with an unchanged τ indicated an increase in S. No direct measurements of DNA synthesis were made, but they showed that the execution point of cdc8 changed from 0.37 of the cell cycle in the control to 0.56 after hydroxyurea treatment, and the execution point of cdc13 (whose gene product is required for nuclear division) changed from 0.35 to 0.5. This was strong indirect evidence for a lengthening of S. In the treated culture start shifted from 0.3 of the cell cycle to 0.0. The pre-start period was greatly reduced until it occupied only the period from mitosis to cell separation. The interval from cdc13 to mitosis was the same in the control and treated cultures, indicating a constant interval from completion of S to mitosis.

In this parallel piece of work Singer and Johnston also found that increasing S, without altering τ, caused an increase in B, and a decrease in G_1. There were major differences, they reported G_2 remaining constant and a significant increase in the period from start to division. However the same general picture was reported, an increase in S, with an unaltered τ, being compensated for by a decrease in other cell cycle phases.

The Role of G_1 and G_2

The decrease in G_1 when DNA replication is slowed down supports to some extent the views of Cooper (1979) and Liskay (1978) that this phase is present because events specific to growth have not yet occurred in sufficient quantities. However there was still a significant G_1 in both parents and daughters, and at the lower concentrations the increase in S caused the requirement of some growth-related event not necessary when 5'-dTMP was present in excess.

The shortening or lengthening of cell cycle phases need not mean that events which normally occur in this phase must be omitted or delayed, or that cell cycle specific events do not occur in this phase (Figure 23).

If a cell cycle pathway was independent of S, an event which normally occurs late in G_1 could occur early in S if this was increased and G_1 decreased (Figure 23b). Events which were growth dependent in a normal G_1 could take place in less time under conditions where less growth was necessary in G_1, e.g. under 5'-dTMP limitation (Figure 23c and d). As a result of the disruption in normal cell cycle events caused by a lengthening of S, a process may be completed earlier (or later) in the cell cycle and may recommence in a different part of the cycle(Figure 23d).

The decrease in G_2 observed was more unexpected. The function of G_2 is probably to synthesize the proteins necessary for chromosome

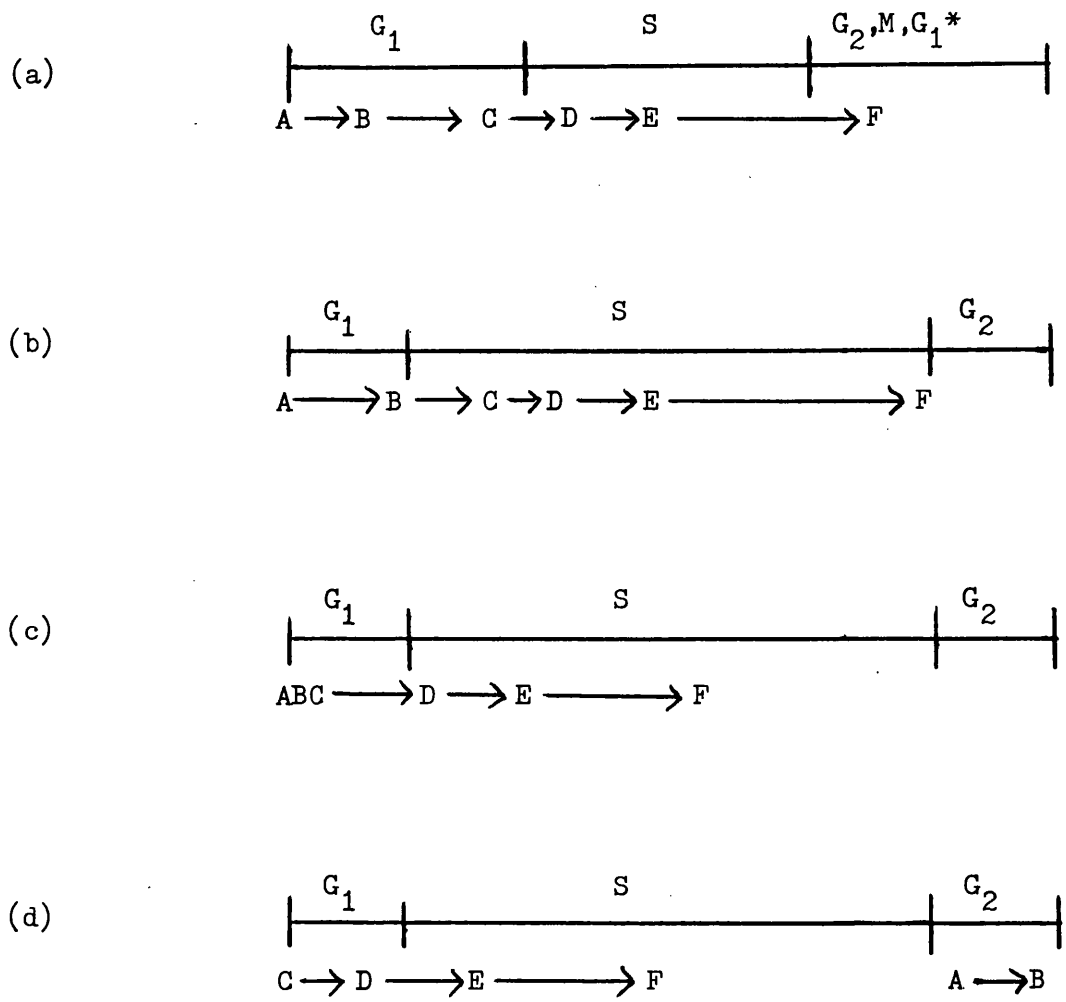


Figure 23. Effect of altering S on other controlled processes.

(a) Control A→B etc are a series of events initiated by an unknown cellular event

(b) Events A→B etc are independent of S

(c) Events A⟶D are growth dependent

(d) Events A⟶B are initiated in previous cycle by the completion of some unknown cellular or growth event.

condensation, and factors to counteract the induction of DNA synthesis. The increase in B with increasing growth rate led Pringle and Hartwell (1981) to propose that the rate of accumulation of some stage-specific components not present in excess can be rate-limiting under conditions of slow growth. It is more likely that this occurs in the flexible G_2 period rather than the more rigid S or M phases. Nuclear division is dependent upon DNA synthesis (Hartwell, 1974) and if events causing this dependency are initiated early in S, under normal conditions G_2 will be x minutes, but if S is elongated they may give a G_2 of x minutes minus the increase in S. This is clearly an over-simplification, if it is applied to the data in Table 17 a negative value is obtained. It is more probable that when cell cycle phases are altered a combination of b, c and d all occur. There seems to be a minimum G_2 period of approximately 20 minutes.

Effect of 5'-dTMP Limitation on Growth and Morphology

When cells growing exponentially in medium N plus 5'-dTMP were removed, washed and added to medium N without 5'-dTMP, after an approximate 66% increase in cell number, division ceased, although growth continued. The increase was presumably caused by cells post-S or cells in S with sufficient internal 5'-dTMP to complete a round of DNA synthesis. Both types of cell would be unaffected by removal of 5'-dTMP from the growth media and would complete their DNA-division cycle.

Approximately half of the cells assumed "Thymine-less death"

morphology and arrested as doublets characteristic of mutants for DNA synthesis or with a thymidine requirement (Game, 1976). The rest were mostly cells with an elongated tubular bud (or buds). The buds were often two or three times normal size, sometimes with invaginations along their length, as if initiation of a new bud had occurred at the same isthmus (Figure 12a).

This morphology is not characteristic of any other cell cycle mutants, although cdc4 arrests with multiple buds. In this mutant spindle plaque duplication occurs, but there is a block in the initiation of DNA synthesis. Despite the absence of DNA synthesis several buds are initiated at regular intervals. Normally there is no new bud formed before cytokinesis (Hartwell, 1974) and it is thought that bud emergence is normally switched off by some event following the initiation of DNA synthesis, which then becomes re-activated at the time of spindle pole body duplication.

Possibly, when mutant 1D was deprived of exogenous 5'-dTMP, in some cells DNA synthesis either progressed extremely slowly, or had stopped prior to the event which switched off the budding mechanism. In the absence of normal DNA synthesis localised growth occurred at the bud isthmus and new buds were initiated.

At reduced 5'-dTMP concentrations, when thymidine was limiting for DNA synthesis there was an increase in the percentage of cells with aberrant morphologies (Table 8). At non-limiting concentrations, less than 1% were abnormal, at 15 µg/ml 10% were abnormal,

and at 7.5 μg/ml (where τ increased), 18% were abnormal. Three major classes of abnormalities were identified, all of which increased with decreasing 5'-dTMP concentration. Firstly, cells showing secondary bud formation, secondly, cells with their buds in the centre of a "bean-shaped" cell, and thirdly, the major class, cells that were elongated, either parent, daughter or both (Figure 12).

Secondary bud formation occurs when a new cycle is initiated before cell separation has been completed (Chapter 1). The elongation of S may allow some growth events, which under non-limiting concentrations occur in the unbudded G_1 period, to take place during the elongated budded period, and a new cycle can be initiated earlier, in some cases before completion of the previous DNA-division cycle and this is manifested by the presence of buds before cell separation.

On the "bean-shaped" cells, buds emerged at the middle of the convex side. The morphology may have arisen if a bud was initiated at the tip of the cell but failed to develop properly, if the next bud was then initiated in the correct position this would now be the centre of the cell. Alternatively, the abnormal shape could be due to errors made in cell wall composition during bud growth.

Low 5'-dTMP concentrations also caused some cells to lose their normal prolate spheroid shape and become elongated. In a normal budded period the alkali-insoluble glucans, which help to

maintain yeast-shape, are deposited at the latter stages of bud formation (Matile, Moor & Robinow, 1968). The increase in the budded phase, with an unchanged τ, may result in more glucans being deposited, giving a more rigid tubular shape to the daughter cell.

Decreasing the thymidine concentration also affected the volume at bud initiation for cells of all genealogical ages. At 50 µg/ml the volume of daughters at bud initiation was 27 μm^3, at 15 µg/ml it was 40 μm^3 and at 7.5 µg/ml the volume more than doubled compared to non-limiting values, to 65 μm^3. The increase is difficult to explain. The lengthening of the budded phase at the lower concentrations was compensated for by a decrease in the unbudded phase, and as growth has been shown to be exponential during the cell cycle (Hayashibe, 1977), volume at bud initiation should be unaltered. The 50% increase in cycle time at 7.5 µg/ml only partly explains the 130% increase in volume. Lengthening of S phase, caused by 5'-dTMP limitation, caused a substantial increase in cell volume at bud initiation through some unknown metabolic effect.

Increasing the S phase by 5'-dTMP limitation as well as affecting the length of other cell cycle phases also affected growth and morphology. B was lengthened, cell volume at bud initiation was increased, secondary bud formation was seen and some cells lost their normal prolate spheroid shape. Presumably this was because at the lower concentrations cells were unable to take up sufficient 5'-dTMP to support a normal length round of DNA

replication. This idea was supported by the results of 5'-dTMP limitation in mannose and YEPD medium.

The Influence of Cell Cycle Events on Growth and Morphology

The work with the chemostat and the 5'-dTMP mutants have shown that alterations in the timing of cell cycle events can significantly affect growth and morphology.

cdc4 shows that bud emergence is regulated by DNA synthesis (see above). A number of genes defective in nuclear division do not undergo cell separation or bud initiation, and nuclear division must be a prerequisite for these two processes (Hartwell, 1974). This shows that cell cycle genes do regulate some morphological events. Alterations in the growth rate can affect cell shape. An increase in τ caused an increase in B and a decrease in volume (Figures 1 and 7). At very fast growth rates in the chemostat a small percentage of cells exist as filaments (Figure 9). In this case the signal for cell separation was switched off, cells also lost their normal shape and became more cylindrical.

Changes in the timing of cell cycle phases, with an unchanged τ, also affected growth and morphology as the results with thymidine limitation showed (see above).

Some species of fungi exhibit dimorphism - they can exist in either the mycelial habit or a yeast-like unicellular morphology,

depending upon environmental conditions. It is believed that the change is brought about structurally by differential rates of growth of different regions of the cell wall (Stewart & Rogers, 1978).

Conditions favouring the yeast form include, an anaerobic environment, fermentative growth, high glucose or other sugar concentrations and high temperatures. Mycelial form is favoured by an aerobic environment, respiratory growth and low sugar concentrations. Cyclic AMP is also involved, low concentrations allowing yeast-like growth, high concentrations mycelial. Yeast form is associated with conditions pertaining to fast growth rates and the mycelial form to slow growth rates (Stewart & Rogers, 1978). A decrease in the growth rate may affect some part of the DNA-division cycle, the changes in which may then influence cell wall formation, the means by which it is believed a change of form is achieved. Whatever the cause, growth rate is clearly involved in the regulation of dimorphism and the need to examine events of the DNA-division cycle as well as biochemical and metabolic events is apparent.

CHAPTER FIVE

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ACKNOWLEDGEMENTS

I am particularly grateful to Dr. A. E. Wheals for his help and guidance throughout the course of this project. I wish to thank my wife Julie, for all of her support, my parents for their financial help during my education, and my colleagues in the Microbiology Department of the University of Bath for their assistance, advice and friendship.

The work was carried out under the tenure of an S.R.C. studentship.